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A STUDY OF THE METABOLISM OF CRYSTALLINE PROGESTERONE

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In 1938 (1) a preliminary report was made on a study of the metabolism of injected crystalline progesterone, which definitely established sodium pregnandiol glucuronidate as an end product in the metabolism of this compound in the human organism. These experiments have been elaborated and are now being reported in detail.

Since this work was published Stover and Pratt (2) reported failure to demonstrate any sodium pregnandiol glucuronidate in the urine of women following injections of 15 and 17.5 RB. U. of progestin (Parke, Davis). Also Hamblen and coworkers (3, 4) reported that in their studies on progesterone utilization in patients with functional irregularities of uterine bleeding no evidence was encountered that crystalline progesterone was metabolized into sodium pregnandiol glucuronidate. From endometrial studies they also conclude that progesterone is inefficiently utilized in these cases. Recently in an advance abstract (19) they state they were only able to recover a total of 3 mg. and 5 mg. of sodium pregnandiol glucuronidate from the urine of 2 patients who had received 90 mg. and 200 mg. of progesterone respectively. In a recent paper Seegar (20) reported that endometrial hyperplasia may respond to progesterone and show changes resembling the physiologic response. The amount of progesterone excreted in the urine as pregnandiol when 50 mg. in 5 mg. doses is given daily, is insufficient to be recovered consistently in measurable quantities in these cases. On the other hand Buxton and Westphal (5) recovered sodium pregnandiol glucuronidate in the urine of males following large doses of crystalline progesterone.

In the present study the excretion of sodium pregnandiol glucuronidate was followed under various conditions, and in many cases an attempt was made to correlate this excretion with simultaneous changes occurring in the endometrium. Injections of progesterone were given in the follicular and luteal phase of the menstrual cycle, during pregnancy, after hysterectomy, in cases of secondary amenorrhea with hypoplastic endometria with or without pretreatment with estrogens and in cases of metropathia hemorrhagica with hyperplasia of the endometrium. The effect of estrogens and of gonadotropic hormone upon the excretion of sodium pregnandiol glucuronidate was also investigated.

The determination of sodium pregnandiol glucuronidate was made according to the method of Venning (6, 7). There are several points which should be emphasized regarding the estimation of this compound. Firstly, it is impossible to detect less than 2 mg. in a sample of urine, therefore 2 or more days must be pooled and extracted together in order not to overlook small amounts. Secondly, unless care is taken in

keeping the specimens cold and in using a suitable preservative, considerable hydrolysis occurs with loss of sodium pregnandiol glucuronidate. Thirdly it is always necessary to identify small amounts of residue by means of a melting point in order to distinguish the glucuronide from possible impurities. In reporting the results, the weight of sodium pregnandiol glucuronidate excreted is converted by means of a factor into terms of mg. of pregnandiol and expressed as such. In this manner it is easy to compare amounts of pregnandiol with corresponding amounts of progesterone from which it is derived as the molecular weights of the two compounds are almost identical.

Crystalline progesterone[1] was given intramuscularly in the form of a solution in sesame oil and estrogen as estradiol benzoate. The gonadotropic hormone physex (Leo Chem. Co.) used is a chorionic gonadotropic hormone preparation in the form of a dry powder extracted from human pregnancy urine.

EXPERIMENTAL

Case 1. E.R. (aged 33 years, table 1). A supravaginal hysterectomy had been performed 10 days before the injections were begun. Eight mg. progesterone were injected daily on the second, fourth and fifth days of the experiment, a total of 24 mg. The daily urinary specimens were examined separately for pregnandiol. None was detected. On the eighth day 20 mg. sodium pregnandiol glucuronidate was injected daily for 3 days. The amounts excreted are shown in table 1. A total of 34.8 mg. or 58% was recovered. On the 14th, 15th and 16th days 10 mg. pregnandiol was injected. None could be detected in the urine.

Case 2. M.S. (aged 51 years, table 1). A total hysterectomy and oöphorectomy was performed 9 days before the experiment. Twenty-four mg. progesterone was injected over a period of 4 days. The daily urinary specimens were assayed separately. No pregnandiol was recovered. Three days later 20 mg. sodium pregnandiol glucuronidate was injected daily for 3 days. The daily excretion of this substance was 7.0, 7.8, 9.5 and 1.5 mg. respectively, making a total amount of 25.8 mg. and a recovery of 43%. Ten mg. pregnandiol was injected on the 14th, 15th and 16th days. None, however, was excreted in the urine.

Case 3. Q.B. (aged 37 years, table 1). A supravaginal hysterectomy and right salpingo-oöphorectomy had been performed 3 weeks previously. Thirty mg. progesterone was injected daily for 3 days (a total of 90 mg.); 4.6 mg. pregnandiol was excreted during the first 2 days and 4.0 during the next 3 days, making a total of 8.6 mg. This represents a recovery of 9.6% of the injected progesterone. Apparently if a sufficiently large dose of progesterone is given, the body is able to metabolize it to pregnandiol in the absence of the uterus.

Case 4. F.R. (aged 34 years). A bilateral salpingo-oöphorectomy had been performed 3 years previously because of large ovarian cysts, menopausal symptoms followed and estrin therapy was given. At the time of observation she had been receiving injections of 10,000 I.U. estradiol benzoate every 2 weeks. Ten mg. progesterone was injected daily for 3 days. A total of 3.6 mg. pregnandiol was excreted in the pooled specimens of urine, making a recovery of 12%.

Case 5. E.G. (aged 23 years). This patient had abnormally long menstrual cycles 60 to 90 days apart, and progesterone was injected during the follicular phase. Endometrial biopsies taken on the 13th and 20th days showed early proliferation. Beginning on the 30th day, 10 mg. progesterone was given daily for 5 days. No pregnandiol was excreted during the first 2 days of the injections, 1.2 mg. daily was recovered in the pooled specimens of the last 3 days. A biopsy taken on the last day of the injections showed early progestational changes, numerous glands with vacuolization at base of cells and beginning edema in stroma. Following discontinuance of the progesterone injections, bleeding occurred on the 36th day.

Case 6. N.I. (aged 36 years, fig. 1). This case has a normal menstrual history and the excretion of pregnandiol had been followed by three cycles prior to this experiment. The length

[1] The crystalline progesterone used in these experiments was partly synthesized in our laboratory and partly supplied by Dr. Schwenk of the Schering Corporation. We are also greatly indebted to Dr. Schwenk for a generous supply of progynon-B.

of the cycles varied from 25 to 27 days. The total amount of pregnandiol excreted ranged from 52 to 55 mg. over a period of 10 to 12 days. Figure 1 represents 4 separate menstrual cycles. Cycle 1 shows the output of pregnandiol in the average cycle. In cycle 2 10 mg. of progesterone was injected on the 19th and 20th days, at a time when pregnandiol was already being excreted normally. There was an increased output of pregnandiol approximating 20% of the injected progesterone. In cycle 3 estrin and progesterone were injected simultaneously during the luteal phase. Collections were begun on the 16th day of the cycle, the excretion of pregnandiol at that time showed a normal level for this particular case. On the 18th day 10 mg. progesterone and 10,000 I.U. estradiol benzoate were given and on the 19th day 20 mg. progesterone and 10,000 I.U. estradiol benzoate. Instead of an increased excretion of pregnandiol occurring as in the previous cycle, there was actually a decreased output and bleeding occurred earlier, the cycle having been shortened to 21 days. In the 4th cycle, 2 single injec-

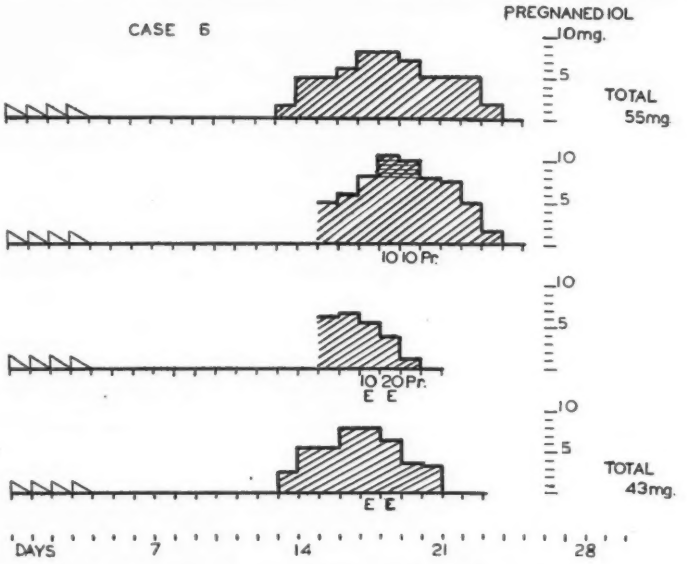

Fig. 1. Excretion of pregnandiol in *case* 6, normal menstrual cycle.
PR—progesterone. E—10,000 I.U. estradiol benzoate.

tions of 10,000 I.U. estradiol benzoate were given on the 18th and 19th days. Again the pregnandiol excretion tended to fall off more rapidly than normally, following the estrin injections. The luteal phase was shortened to 8 days and the cycle to 23 days, the total output of pregnandiol being decreased to 43 mg. In another cycle not illustrated after a single injection of 10 mg. progesterone on the 9th day of the cycle no excretion of pregnandiol was detectable.

Case 7. *N.H.* (aged 42 years, a normal case, fig. 2). Two cycles were followed for the excretion of pregnandiol. The first shows the normal output of pregnandiol. In the second cycle 2 injections of 10 mg. progesterone were given at the beginning and 2 injections of 20 mg. each at the end of the luteal phase. There was an increased total excretion of pregnandiol as compared with the previous cycle 65.3 mg. as against 43.1 mg. a difference of 22.2 mg. The shape of the curve is different as seen in figure 2. There is a greater daily excretion of pregnandiol at the beginning and end of the luteal phase.

Case 8. *N.B.* (aged 26 years, complained of acne, fig. 2). This patient had previously been studied and had a normal luteal phase, the total output of pregnandiol being 60 mg. over a period of 13 days. In the next cycle 100 M.U. each of antex (pregnant mare's serum) and

physex (pregnancy urine extract) were given on the 12th, 13th and 14th days of the cycle. Figure 2 shows the excretion of pregnandiol throughout this cycle. Three injections of 8 mg. progesterone were given on the 24th, 25th and 26th days. There was a definite increase in the excretion of pregnandiol following these injections, approximately 55% of the injected progesterone.

Case 9. W. (aged 39 years, fig. 2). (This patient normally has regular cycles. The total excretion of pregnandiol has varied from 24 to 45 mg. over a period of 6 to 10 days. In the

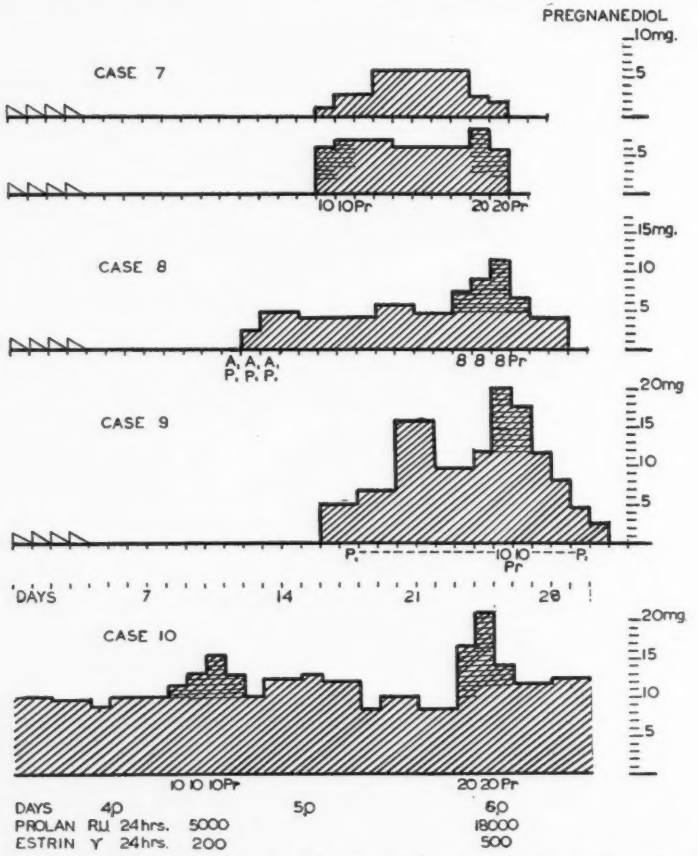

Fig. 2. Excretion of pregnandiol in *cases* 7, 8, 9, menstrual cycles and *case* 10, early pregnancy. PR—progesterone. A—100 M.U. antex. P—100 M.U. physex.

particular cycle illustrated in figure 2, pregnancy urine extract was given throughout the luteal phase (100 M.U. physex daily). There was a rapid increase in the output of pregnandiol following the injections of gonadotropic hormone and the luteal phase was considerably lengthened. Ten mg. progesterone was injected on the 26th and 27th days of the cycle after the level of the pregnandiol had fallen. These injections caused a second rise in the excretion of pregnandiol. The increased excretion represented an approximate recovery of about 70% of the injected progesterone.

Case 10 R.C. (aged 31 years, fig. 2). A case of normal pregnancy during which two series of injections of progesterone were given. Ten mg. was injected for 3 days on the 44th, 45th

and 46th days of pregnancy and later 20 mg. were given on the 59th and 60th days. In both instances there was a definite rise in pregnandiol excretion immediately following the injections. The first increase in excretion represented 38% of the injected progesterone, and the second, about 50%. On discontinuance of the progesterone, the excretion of pregnandiol fell back to its normal level.

We have found that in early pregnancy, pregnandiol is excreted at an unusually even

TABLE 1

Days	Injections of progesterone	Other injections	Pregnandiol excreted	Recovery
	mg.	*mg.*	*mg.*	
Case 1				
1	0		0	
2	8		0	
3	0		0	
4	8		0	
5	8		0	0
6–7	0		0	
8		20 Na pregnandiol gluc.	7.4	
9		20 Na pregnandiol gluc.	8.6	
10		20 Na pregnandiol gluc.	13.3	
11		—	5.5	58
12		—	0	
13		—	0	
14		10 Pregnandiol	0	
15		10 Pregnandiol	0	
16		10 Pregnandiol	0	
17–18		—	0	
Case 2				
1	0		0	
2	8		0	
3	0		0	
4	8		0	
5	8		0	0
6–7	0		0	
8		20 Na pregnandiol gluc.	7.0	
9		20 Na pregnandiol gluc.	7.8	
10		20 Na pregnandiol gluc.	9.5	
11		—	1.5	43
12–13		—	0	
14		10 Pregnandiol	0	
15		10 Pregnandiol	0	
16		10 Pregnandiol	0	
17–18		—	0	
Case 3				
1	0		0	
2	30		4.6	
3	30			
4	30		4.0	
5	0			
	Total 90 mg.		Total 8.6 mg.	9.6

rate. This makes it possible to calculate the recovery of added progesterone more accurately than in the menstrual cycle where the level of pregnandiol excretion is constantly changing. The prolan and estrogen excretion at these two periods are marked on the chart.

Case 11 E.W. (aged 20 years, table 2). This patient had had regular menstrual periods up to the age of 17 years. Since then they had been irregular, and at the time of observation she had been amenorrheic for one year. A curetting showed a hypoplastic endometrium. She was given 200 M.U. of gonadotropin derived from mare's serum (antex) and 100 M.U. chorionic gonadotropin (physex). Assays were carried out on the urine. No pregnandiol was excreted

for 10 days after these injections. Beginning on the 11th day 3 daily injections of 8 mg. of progesterone were then given. No pregnandiol could be detected in the urine and no bleeding followed. She was then given 10,000 I.U. estradiol benzoate daily for 11 days. This was followed by 5 daily injections of 10 mg. progesterone. A total of 6.0 mg. of pregnandiol were excreted in the urine over this period, making a total recovery of 12% of the injected progesterone in the form of pregnandiol. An endometrial biopsy, taken on the last day of the injections showed only a very early progestational effect with vacuolization at the base of the cells. Bleeding occurred on the day following this biopsy. After 4 months no spontaneous periods had occurred, 7 daily injections of 10,000 I.U. estradiol benzoate were then administered, this was followed by 6 daily injections of 10 mg. progesterone. Three more injections of estradiol benzoate were also given during the period of progesterone treatment. A total of 10.8 mg. of pregnandiol was excreted in the urine corresponding to an 18% recovery of the injected progesterone. A biopsy taken on the day following the last injection of progesterone showed a somewhat more advanced progestational effect in the endometrium than on the previous occasion. There was secretion in some of the glands and beginning edema in the stroma. Bleeding occurred on the third day after discontinuance of progesterone.

TABLE 2

Days	Injections of progesterone	Other injections	Pregnandiol excreted	Recovery
	mg.		mg.	%
Case 11				
1		100 M.U..A[1]	0	
2		100 M.U.A + 100 M.U. P[1]	0	
3–10		—	0	
11	8		0	
12	8		0	
13	7		0	
14	0		0	
Total	24		0	0
15–23		0.333 mg. E.B.[1]×9		
24	10	0.333 mg. E.B.		
25	10		4.6	
26	10			
27	10			
28	10		1.4	
Total	50		6.0	12
4 months later				
1–9		0.333 mg. E.B.×10	0	
10	10			
11	10	0.333 mg. E.B.	3.4	
12	10			
13	10	0.333 mg. E.B.	3.0	
14	10			
15	10	0.333 mg. E.B.	4.4	
Total	60		10.8	18
Case 12				
1–11		0.333 mg. E.B.×6		
12–16	10×5		No assays	
17	10		2.4	
18	5			
19	10		2.8	
20	10	100 M.U. P.		
21	10	100 M.U. P.	4.3	
22	10	100 M.U. P.		
Total	105		9.5	17.2

TABLE 2.—*Continued*

Days	Injections of progesterone	Other injections	Pregnandiol excreted	Recovery
	mg.		*mg.*	%
5 months later				
1	10			
2	10		0	
3	10			
4	10			
4	10			
5	10		1.2	
6	10			
7	10			
Total	70		1.2	1.7
Case 13				
1–11		0.333 mg. E.B.×6	0	
13	5		1.6	
14	5			
15	5			
16	5		3.0	
17	5			
Total	25		4.6	18.4

[1] M.U. A. Mouse units antex.
M.U. P. Mouse units physex.
E.B. Estradiol benzoate.

Case 12 M.L. (aged 24 years, table 2). At the time of observation this patient had been amenorrheic for 7 months. Two periods of bleeding occurred after estrogen therapy. In the following month 8 injections of 10,000 I.U. estradiol benzoate, one every other day, were given. This was followed by 11 daily injections of progesterone of 10 mg. each, excepting on the 7th day when only 5 mg. were injected, making a total amount of 105 mg. progesterone. Collections of urine were only made during the last 6 days of progesterone injection, however. On the last 3 days of the experiment 100 M.U. luteinizing hormone (physex) were given daily, a total of 9.5 mg. of pregnandiol were excreted during the last 6 days of the experiment during which time 45 mg. of progesterone had been injected. This represents a recovery of 17%. A biopsy taken at the end of the experiment showed edema and well advanced vascularization of the stroma. The stroma had a predecidual appearance, the glands were dilated, with cuboidal epithelium. Bleeding occurred within 48 hours of the biopsy. During the next 3 months, periods of bleeding followed the use of monthly injections of physex. One hundred M.U. physex was given daily for 3 days about the 14th day after the beginning of the last menstrual period and bleeding occurred within 7 to 14 days after cessation of injections. In May, however, no bleeding occurred following these injections. Twenty-eight days later (45 days from the last period of bleeding) 7 injections of 10 mg. progesterone were given. No pregnandiol was excreted during the first 3 days. A questionable trace of pregnandiol was found in the pooled specimens of urine of the last 4 days. A biopsy taken at the end of the injections showed practically no effect on the endometrium. Most of the glands and stroma were in the proliferative state. Only in an occasional gland was there some suggestion of lining up of the nuclei and vacuolization at base of cells. Bleeding followed, however, after cessation of injections.

Case 13. S. (aged 32 years, table 2). This was a case of secondary amenorrhea with hypoplasia of the endometrium. At the time of the experiment, she had been amenorrheic for 6 months. She was given 6 injections of 10,000 I.U. estradiol benzoate every other day followed by 5 injections of 5 mg. progesterone. Assays were carried out on the pooled urine. A total of 4.6 mg. pregnandiol was excreted during the 5 days representing a recovery of 18.4%. No biopsy was taken and bleeding occurred within 3 days after the last injection of progesterone.

Case 14. C. (aged 34 years). The patient complained of very long and irregular cycles. A biopsy taken 30 days after a period of bleeding showed a moderate degree of hyperplasia in the endometrium. Following a period of amenorrhea of 2 months, 7 daily injections of progesterone were given, 4 of 9 mg. and 3 of 10 mg. making a total of 66 mg. No pregnandiol was excreted during the first 3 days, 2.8 mg. was recovered from a pooled specimen of urine during the last 4 days. A biopsy taken at the end of the experiment showed numerous cystic glands with patchy progestational transformation. Bleeding occurred 2 days after the last injection.

Case 15. G.R. This patient, a case of secondary amenorrhea in a married woman aged 34 years, had irregular bleeding for the past 10 years. Following a period of 10 months amenorrhea, a curetting showed a hyperplastic endometrium with cystic dilatation of the glands. Bleeding occurred after the biopsy was taken and 16 days later 10 mg. of progesterone were injected daily for 11 days. No pregnandiol was excreted during the first 6 days, about 1 mg. daily was excreted during the last 5 days. Endometrial biopsies were taken on the fourth and eleventh days of the injections. The one on the fourth showed an endometrium with very dense stroma and irregular and dilated glands with beginning vacuolization at base of epithelial cells but no mobilization of the nuclei. In the biopsy on the 11th day, the glands were more numerous and tortuous, the epithelial cells tended to become cuboidal, the nuclei being arranged in a single row at the base of the cells. There was edema and vascularization of the stroma.

Case 16. S. (table 3). A woman aged 46 years, had metropathia hemorrhagica. A therapeutic curettage had been performed 10 days before the onset of the present bleeding and showed a hyperplastic endometrium. Beginning on the 3rd day of bleeding, 10 mg. progesterone was given daily for 8 days. Bleeding ceased on the 5th day of injections. Pregnandiol

TABLE 3

Days	Injections of progesterone	Other injections	Pregnandiol excreted	Recovery
	mg.		mg.	%
Case 16				
1	10		2.8	
2	10			
3	10		0	
4	10			
5	10		3.6	
6	10			
7	10		2.2	
8	10			
9–10	—		0	
Total	80		8.6	10.7
2 weeks later				
1	10		0	
2	10			
3	10			
4	10		1.0	
5	10	100 M.U. P.	5.0	
6	10	100 M.U. P.		
7	10	100 M.U. P.	2.6	
Total	70		8.6	12.3
18 months later				
1	10		1.8	
2	30			
3	30		12.0	
4	30			
Total	100		13.8	13.8

TABLE 3—*Continued*

Days	Injections of progesterone	Other injections	Pregnandiol excreted	Recovery
	mg.		*mg.*	%
Case 17				
1	10		1.2	
2	10			
3	10		5.2	
4	10			
5	10			
6	10			
Total	60		6.4	10.6
45 days later				
1		200 M.U. P.	0	
2		—	0	
3–5		100 M.U. P.×3	0	
6–8		100 M.U. P.×3	0	
9	10	100 M.U. P.	3.0	
10	10			
11	10		5.8	
12	10			
Total	40		8.8	22.0
Case 18				
1	10		0	
2	10			
3	10		2.0	
4	10			
5	10	100 M.U. P.	5.0	
6	10	100 M.U. P.		
Total	60		7.0	11.6
Case 19				
1	5		0	
2	5		0	
3	10		0	
4	10	100 M.U. P.	1.8	
5	10	100 M.U. P.	4.8	
6	10	100 M.U. P.		
Total	50		6.6	13.2

was excreted during this period a total of 8.6 mg. being recovered. Two weeks later 10 mg. progesterone was again injected daily for 7 days. One hundred M.U. (physex) chorionic gonadotropin was given with the last 3 injections of progesterone. No pregnandiol was excreted during the first 3 days. In the last 4 days 8.6 mg. was recovered in the urine. Bleeding occurred within 48 hours after the last injection. Eighteen months later, about 38 days following a spontaneous menstrual period 4 injections of progesterone were given of 10, 30, 30, 30 mg. respectively. During the first 2 days 1.8 mg. pregnandiol was excreted and 12 mg. during the next 3 days, making a total of 13.8 mg. and recovery of 13.8%. A biopsy taken on the 6th day of progesterone injections showed an early progestational effect, dilated glands, lining up of nuclei and vacuolization at base of cells. Very little active secretion was present.

Case 17. McL. (aged 39 years, table 3). Two years previously this patient had received radium treatment for prolonged and excessive bleeding and subsequently had had estrin therapy for menopausal symptoms after which she menstruated several times without further therapy. Beginning on the 26th day after the last bleeding 6 daily injections of 10 mg. progesterone were given. During the first 2 days 1.2 mg. pregnandiol was recovered in the urine, 5.2 mg. during the last 5 days, representing a recovery of 10.6% of the injected progesterone.

Bleeding occurred on the third day following the discontinuance of progesterone. Forty-five days following this bleeding 100 M.U. physex was given daily for 9 days. No pregnandiol was excreted during these injections. This was followed by 4 daily injections of 10 mg. progesterone. During the first 2 days 3.0 mg. pregnandiol was excreted and 5.8 mg. during the last 2 making a total of 8.8 mg. This represents a recovery of 22%. No biopsies were obtained.

Case 18 A.S. (table 3). A case of metropathia hemorrhagica aged 28 years, who at the time of observation, had been amenorrheic for 6 months. Ten mg. progesterone was injected daily over a period of 6 days. One hundred M.U. physex was given with the last 2 injections. No pregnandiol was excreted during the first 2 days of the injections, 2.0 mg. during the next 2 days and 5.0 mg. during the next 3 days. A biopsy taken on the 4th day showed beginning edema of stroma, numerous dilated glands with vacuolization at base of cells. Bleeding occurred 48 hours after the progesterone injections were discontinued.

Case 19 C. (42 years old, table 3). This patient menstruated only at irregular intervals and could be made to bleed with progesterone alone. Following a period of amenorrhea of 2 months, 6 daily injections of progesterone (2×5 mg.) (4×10 mg.) were given. One hundred M.U. each of physex was given with the last 3 injections. No pregnandiol was excreted during the first 3 days, 1.8 mg. on the 4th day and 2.4 mg. on the 5th and 6th days. Bleeding occurred 2 days after discontinuance of injections.

DISCUSSION

Although at first glance there appears to be a wide variation in the metabolism of the injected crystalline progesterone as measured by the excretion of pregnandiol in the cases reported, several deductions may be drawn from a careful study of our findings.

There are many factors which may have an influence on the excretion of pregnandiol (*a*) state of endometrium (*b*) efficiency of the conjugatory mechanism with glucuronic acid (*c*) efficiency of the renal excretion (*d*) rate of absorption and destruction of the injected progesterone (*e*) excretion of pregnandiol in a form other than the glucuronide (*f*) excretion of progesterone in other forms than pregnandiol. With regard to this last Marker and Kamm in a series of papers have reported that intermediary compounds in the reduction of progesterone to pregnandiol are excreted in small amounts. In their studies, however, the proportion of these intermediary compounds to the pregnandiols in human urine is so small as to be almost negligible in the amount of urine taken for the estimation of sodium pregnandiol glucuronidate. It may be recalled that only approximately 10% of injected estrogens are recovered in the urine of human beings under ordinary circumstances. It is known that estrogens are excreted in the feces and it is possible that pregnandiol is also excreted by this route, though there is no experimental evidence for this.

It seems reasonable to expect some destruction of the injected hormone to occur before it reaches its site of action. Even when we inject sodium pregnandiol glucuronidate, a water-soluble substance, only 43 to 58% of the compound is excreted in the urine as shown in *case 1* and *2*. Progesterone on the other hand, is injected in an oily solution and is metabolized into another compound before being excreted, therefore the loss under these conditions would be expected to be even greater. Following injections of 30 mg. of pregnandiol in an oily solution we were unable to detect any excretion of this compound in the urine in 2 cases. We have found by experience, in order to carry out successful recovery experiments in cases of menstrual disorders, that a dosage of 10 mg. of progesterone must be injected daily. The urine specimens should be pooled so that any one analysis contains at least 3 mg. of the excreted compound. This means putting together 2 to 3 days' specimens. It is impossible to detect pregnandiol in separate 24-hour specimens following daily injections of 5 mg. proges-

terone, by the present method. This explains the failure of Hamblen and coworkers to detect any significant excretion of pregnandiol in the cases following daily doses of 5 mg. progesterone, and also our failure to detect pregnandiol in the first 2 cases studied. Even in their later paper (19) they reported giving larger daily doses and being able only to recover very small amounts. Seegar also found that when 5 mg. progesterone was injected daily, it was insufficient to be recovered consistently in measurable quantities in cases of hyperplasia. The state of the endometrium has a direct influence on the metabolism of crystalline progesterone to pregnandiol. Hisaw and Leonard (8) and Allen (9) reported that in the rabbit progesterone has little or no effect on an endometrium that has not been previously acted on by estrogens. Selye (16) on the other hand has shown that very large amounts of progesterone in the spayed rat will produce progestational changes in the endometrium without previous sensitization by estrogens. However, it is well established that where physiological amounts of progesterone are used in the castrate animal a pretreatment with estrogens will enhance the action of progesterone. This fact is borne out in our experiments on human beings with regard to pregnandiol excretion. In the cases *11*, *12* and *13* of secondary amenorrhea with hypoplastic endometria, none or only a trace of pregnandiol could be detected in the urine following the injections of progesterone. However, when a pretreatment of estrogen (60,000 to 120,000 I.U. estradiol benzoate over a period of 10 to 13 days) was given, 12 to 18% of the injected progesterone was recovered as pregnandiol. *Case 12* showed practically no progestational proliferation in the endometrium following 70 mg. progesterone. When estrogen therapy was given prior to the progesterone, progestational changes occurred in the endometrium and pregnandiol was recovered in the urine.

In the cases of metropathia hemorrhagica with hyperplasia of the endometrium such as cases *14*, *15*, *16*, *17*, *18*, it was assumed that the endometrium was already under the influence of estrogen and therefore no added estrogen was given. Here there was a much more varied response to progesterone both as regards the pregnandiol excretion and degree of transformation of the endometrium. This may possibly be accounted for to a great extent upon the varying amounts of estrogen being elaborated in the different cases. In all these cases, however, where endometrial biopsies were obtained and studied, the excretion of pregnandiol was coincident with the progestational changes occurring in the endometrium. However, no correlation could be made between the amount of pregnandiol excreted and the progestational effect. In *case 16* where very large amounts of progesterone were injected, 100 mg. being given over a period of 4 days, the biopsy taken on the 6th day showed only early progestational changes, dilated glands, mobilization of the nuclei and vacuolization at base of cells. This finding suggests a definite time relationship in the sequence of events taking place in the endometrium under the influence of progesterone; increasing the dosage of progesterone to such high levels does not apparently hasten the progestational changes. It may also be noted that in those instances in which series of injections of progesterone were given, the amount of pregnandiol excreted was greater in the latter part of the series. This might suggest that it takes some time for the mechanisms leading to the excretion of pregnandiol to become effective when they are not already operative prior to the injections as in the cases of the luteal phase of the menstrual cycle and in early pregnancy. In the latter the excretion of pregnandiol is immediate.

The difference between the amounts of pregnandiol recoverable in these cases of secondary amenorrhea, following injections of progesterone and those recoverable in the luteal phase of the menstrual cycle or in early pregnancy was very striking. Here the process of conversion of progesterone to pregnandiol was already in action

under presumably ideal conditions, and greater amounts of pregnandiol were recovered when progesterone was given at these times. Cases 6, 7, 8, 9 and 10 show this quite clearly where amounts of pregnandiol ranging from 20 to 55% were recovered. In these particular cases pregnandiol was already being excreted from the metabolism of the patient's own progesterone so that an approximate estimate only can be made of the increased excretion of pregnandiol due to the injections of progesterone. However, in each case a definite rise can be demonstrated following the injections. When progesterone is injected during the follicular phase, smaller amounts of pregnandiol are excreted as shown in cases 5 and 6.

In an earlier paper (10) we reported that the luteinizing hormone when given during the luteal phase not only increased the output of pregnandiol but also lengthened the luteal phase. In several experiments in the present study it was injected simultaneously with crystalline progesterone. The results are not conclusive although the tendency is for a somewhat greater recovery of pregnandiol from injected progesterone to be obtained during the injection of chorionic gonadotropin. This is seen in cases 12, 16, 17 and 19. In the experiment on *case 18*, a pretreatment with physex was followed by a greater excretion of pregnandiol at the time of the progesterone injections. No pregnandiol was excreted during the injections of physex, however, so that the subsequent excretion of pregnandiol can probably not be attributed to the formation of a corpus luteum.

Case 9 demonstrated the very marked excretion of pregnandiol when the luteinizing hormone is given throughout the luteal phase. When crystalline progesterone is injected at such a time there is a rapid increase in the excretion of pregnandiol at the time of injections, amounting to approximately 70% which disappears within 24 hours after the injections. However, as the level of excretion of pregnandiol was changing in this case, it is impossible to evaluate the true recovery.

Injected progesterone is readily metabolized and excreted as pregnandiol in the normal pregnant woman. In *case* 10, pregnandiol was being excreted at a fairly constant level. Following the 2 series of injections given on the 44th and 59th days of pregnancy, there was a definite rise in the output which fell back to the original level at the end of the injections. At that time considerable amounts of gonadotropic hormone were already being elaborated as judged by the excretion of prolan (5000 and 18,000 H.U./24 hr.). The increase of pregnandiol due to the injected progesterone can readily be distinguished and represents a recovery of approximately 38 and 51% respectively.

We have already discussed the necessity of the endometrium being brought to a certain stage of development under the influence of estrogen before it can satisfactorily utilize moderate amounts of progesterone. We were interested to know what effect an excess of estrogen would have on this metabolism. In experiments carried out last year and reported at the International Congress (11, 17) we were able to demonstrate that when added estrin is injected continuously throughout a normal luteal phase, the total amount of pregnandiol is considerably decreased. Hamblen (12) has recently reported a similar effect of estrogen in a case of premenstrual dermatosis. This effect of added estrogen is clearly demonstrated in *case* 6, with regard to the metabolism of added progesterone. When progesterone alone is injected during a normal luteal phase, there is an increased output of pregnandiol, however, when estrogen and progesterone are injected simultaneously there was a sudden falling off in the excretion of pregnandiol with a result of a shortened cycle. In cycle 4 when estrogen alone was given over a short period it had the effect of causing a decrease in the total output of pregnandiol and a shortening of the luteal phase. Zondek (13) has shown that if estrogen is injected beginning on the 21st day of a cycle, premen-

strual changes could be arrested and further development of the corpus luteum prevented. This effect may either be due to a direct action of estrin on the mucosa itself or to an inhibitory effect on the pituitary. Sturgis and Albright (18) have shown that when estrogen is injected in the early part of the menstrual cycle it will inhibit the pituitary and prevent ovulation.

In our earlier experiments on hysterectomized women, cases 1 and 2, we were unable to detect any pregnandiol in the urine following 3 injections of 8 mg. progesterone and suggested that the presence of an uterine endometrium was necessary for the utilization of progesterone. Unfortunately the urine had not been pooled so that the possibility of overlooking small excretions of pregnandiol cannot be excluded. Further experiments revealed, however, that if a sufficiently high dosage of progesterone was given, pregnandiol could be recovered in the urine in such cases. In *case* 3 a supravaginal hysterectomy and right salpingo-oöphorectomy were performed, 3 weeks later 30 mg. progesterone was injected daily for 3 days. The urine specimens were pooled and a total of 8.6 mg. pregnandiol was recovered representing 9.6% of the injected progesterone. In a personal communication in May 1939 Buxton and Westphal (21) state that following 30 mg. progesterone given for 3 days, 3 out of 4 cases of supravaginal hysterectomy excreted 1.1 to 1.9 mg. pregnandiol daily, the fourth patient failed to excrete pregnandiol. In the case of hysterectomy it is possible that other organs acted on by progesterone may convert it to pregnandiol. This may also be so in the case of males.

Although pregnandiol has been found in the urine of other species besides man, so far its excretion as a glucuronide is peculiar to the human race. Marker (15) has reported that the bull excretes twice as much pregnandiol as the pregnant cow. If the testes are removed in infancy, however, this excretion of pregnandiol is prevented. This is interesting in the light of Buxton and Westphal's (5) discovery that the human male was able to metabolize relatively large amounts of progesterone to sodium pregnandiol glucuronidate.

That sodium pregnandiol glucuronidate may also be derived from a source other than the corpus luteum in non-pregnant women appears from the report of Venning, Weil and Browne (14) who found that sodium pregnandiol glucuronidate was excreted in 2 cases of the adreno-genital syndrome although no corpora lutea were present in the ovaries at the time, and the uterine endometrium was atrophic. These findings qualify to some extent the view that the excretion of pregnandiol is invariably an index of production of progesterone and utilization by the uterine endometrium. We are still of the opinion, however, that under normal conditions, the excretion of pregnandiol in the luteal phase may be looked upon as an index of corpus luteum activity. In the numerous cases examined we have never found that sodium pregnandiol glucuronidate was excreted in the urine during the early part of the menstrual cycle. The failure of other workers to find pregnandiol in the urine following injections of progesterone may be attributed to several reasons. (*a*) Failure to use sufficiently large doses of progesterone. (*b*) Failure to pool the urine specimens and to prevent hydrolysis of the compound by the use of a suitable preservative.

It should be remembered that the amounts injected are small and when one considers all the factors involved in its recovery in urine i.e., its absorption and circulation in the body, complete collections of urine and possibility of hydrolysis, it is surprising that these small amounts can be recovered. This study indicates that injected synthetic progesterone is under favorable conditions metabolized as well as intrinsic progesterone and that low recoveries are probably dependent upon the absence of factors normally concerned in the excretion rather than to a difference in the metabolism of injected progesterone and that secreted by the corpus luteum.

SUMMARY

We may summarize our finding as follows.

Pregnandiol can be excreted in hysterectomized individuals when large amounts of progesterone are given.

Cases of amenorrhea will also excrete pregnandiol when large amounts of progesterone are given but the excretion is facilitated by the building up of the endometrium with estrogens.

In cases of metropathia hemorrhagica the recovery is variable. In the above 3 groups of cases the recovery of progesterone improves as the series of injections is continued.

In the 2 groups of cases studied during the luteal phase of the menstrual cycle and normal pregnancy, the recovery of progesterone as pregnandiol is much greater and there is no time lag in the appearance of the increased excretion. From the above it appears that while the endometrium is not essential for the excretion of pregnandiol, the presence of a normal proliferated endometrium facilitates this conversion provided that the other mechanisms involved in the excretion are normal. While sodium pregnandiol glucuronidate can be found in the urine under conditions when all the normal mechanisms involved in its excretion are not present (hysterectomy, atrophic endometrium, failure of conjugation) the recovery of injected progesterone under these circumstances is poor. On the other hand, when all the normal mechanisms are operative (luteal phase of the menstrual cycle, early pregnancy) the recovery is greater and is immediate. Under the latter circumstances there is no difference between the recovery of injected progesterone and injected sodium pregnandiol glucuronidate.

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THE EFFECT OF ESTROGENS ON THE PREGNANDIOL OUTPUT DURING THE MENSTRUAL CYCLE

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IT WAS SHOWN by Zondek (1) that the time of onset of menstrual bleeding may be delayed or in some cases completely inhibited by the injections of estrogen. 100,000 to 200,000 M.U. folliculin given early in the cycle will in most women prevent the next menstrual cycle from occurring and will prevent the formation of a corpus luteum.

Kurzrok and Wilson (2) also found that large doses of estrin will disturb the menstrual rhythm causing a lengthening of the cycle. By means of endometrial biopsies they found that ovulation was postponed for as long as 4 weeks.

On the other hand Clauberg's (3) investigations suggested that doses of 100,000 to 200,000 M.U. of estrin between the 11th to 14th day in single injections will promote ovulation. In one case a dose of 20,000 M.U. was followed by conception in the same cycle in a woman who had been sterile for 4 years.

Zondek (1) further showed that premenstrual changes could be prevented or arrested if estrin were given beginning on the 21st day of the cycle. Biopsies on the 19th, 21st and on the day of the expected menses gave the impression that further progestational development of the mucosa ceased. He offered several possible explanations for these effects. Estrin may hinder the action of progesterone on the mucosa. Estrin may hinder the formation of progesterone, or it may hinder the production of gonadotropic hormone in the anterior pituitary, so that corpus luteum formation, and therefore progesterone production is prevented or further development of an already formed corpus luteum prevented.

He concluded that estrin must be given before the eighth day in a total dose of over 600,000 M.U. if the formation of the corpus lutem was to be prevented. He stated that estrin given even in the last week of the cycle will influence an already formed corpus luteum producing parenchymatous degeneration in that organ, which is visible histologically. On the other hand, Allen and Heckel (4), Westman and Jacobsohn (5) and Klein (6) showed that in the pseudopregnant rabbit the corpora lutea could be preserved by the injection of estrogen. The latter author did not however always observe the persistence of a fully progestational endometrium. Westman and Jacobsohn (7) and Robson (8) were able to repeat this on the hypophysectomized rabbit and claimed that the action of estrogen was a direct one, on the corpus luteum. Westman (9) has recently presented 2 cases tending to show that estrogen will prolong the corpus luteum function in the human female.

In the present study corpus luteum function was estimated by the amount of pregnandiol in the 24-hour volume of urine, using the technic described by Venning (10). Estrin was administered intramuscularly in the form of estradiol benzoate.[1]

Case 1. (Age 25 years, fig. 1.) Menses were 14×26–27×4–5 average flow. This patient

[1] We are indebted to Dr. E. Schwenk of the Schering Corporation for a generous supply of this material.

suffers from a very severe dysmenorrhea. Pain begins one hour after onset of bleeding and is situated in the lower abdomen and legs, lasting approximately 12 hours and necessitating morphine. Pregnandiol extractions were carried out with the following results.

Cycle 1. No therapy, length of cycle 24 days, total pregnandiol recovered 50.8 mg. over 11 days (13–24th) of the cycle.

Cycle 2. Progynon-B, 10,000 I.U. given on the 16th, 18th, 21st, 23rd days, length of cycle 24 days. Total pregnandiol recovered 32.5 mg. over 9 days (15th to 24th) of the cycle.

Cycle 3. Progynon-B, 10,000 I.U. given on the 14th, 16th, 18th, 20th 22nd, 24th, 26th days, length of cycle 27 days, total pregnandiol recovered 30.2 mg. over 11 days from the 15th to 26th days of the cycle.

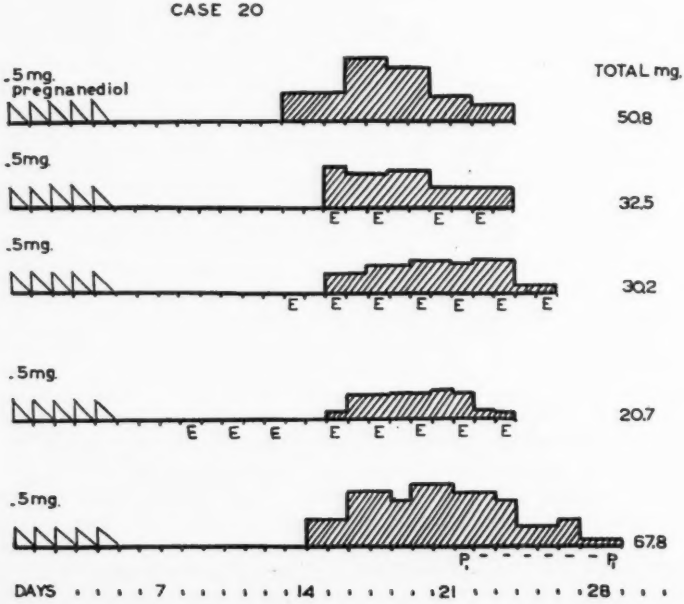

Fig. 1. EXCRETION OF PREGNANDIOL DURING SEVERAL MENSTRUAL CYCLES IN A CASE OF DYSMENORRHEA, TREATED WITH ESTRADIOL BENZOATE AND CHORIONIC GONADOTROPIN. The serrated areas 'E' indicates 10,000 I.U. estradiol benzoate, given intramuscularly. P_1 indicates 100 M.U. physex (chorionic gonadotropin). Length of lines indicate length of cycle.

Cycle 4. Progynon-B, 10,000 I.U. given on the 9th, 11th, 13th, 16th, 18th, 20th, 22nd and 24th days, length of cycle 25 days, total pregnandiol recovered 20.7 mg. over 9 days (15th to 24th) days of the cycle.

Cycle 5. Physex (pregnancy urine gonadotropic substance) 100 M.U. intramuscularly, given daily from the 21st to the 29th days, length of cycle 30 days, total pregnandiol recovered 67.8 mg. over 16 days, 14th to 30th days of the cycle.

Case 2. (Age 32 years, fig. 2.) In 1931 this patient began to have single clots passed midway between the period. A polyp was removed. The intermenstrual bleeding has gradually increased in severity. At present it starts 5 days after cessation of the period (11 days after the onset) and continues for 10 days. For the past year there has been pain in the back associated with both the true menstrual flow and intermenstrual bleeding.

Cycle 1. No therapy, length of cycle 28 days. Total pregnandiol recovered 43.8 mg. over 9 days from the 17th to 26th days of the cycle.

Cycle 2. Progynon-B, 10,000 I.U. given on the 11th, 12th, 13th days, no pregnandiol

was recovered in this cycle, although there was continued spotting from the 13th day on which may have obscured small amounts of pregnandiol. Operation on the 26th day showed both ovaries filled with small cysts, the right ovary showed a yellow colored area which

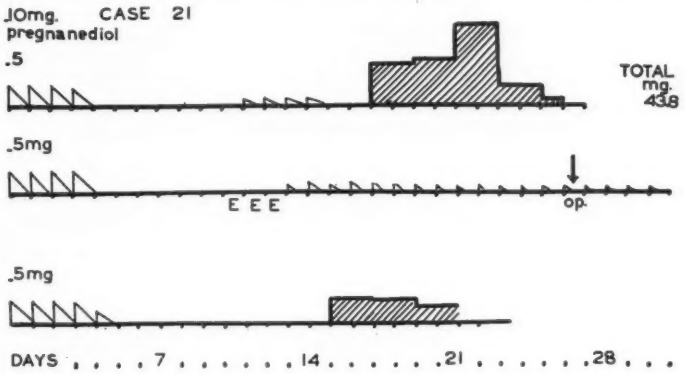

Fig. 2. EXCRETION OF PREGNANDIOL IN A CASE OF INTERMENSTRUAL BLEEDING. Serrated areas indicate bleeding, 'E' indicates injections of 10,000 I.U. estradiol benzoate given intramuscularly. OP indicates laparotomy.

was excised; section (fig. 3) showed it to be a follicle with thecal luteinization, but with no luteinization of the granulosa. Though several sections were made no point of rupture was found in those examined and it is impossible to state whether the follicle had ruptured or not.

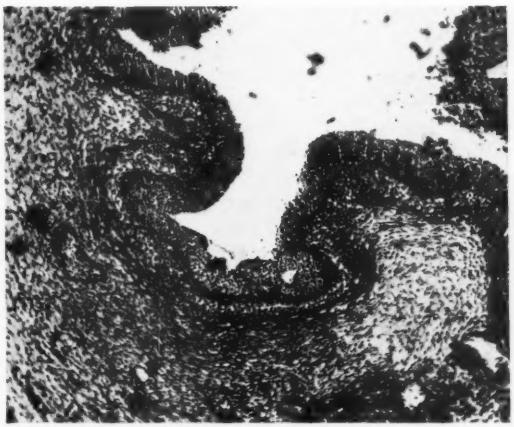

Fig. 3. SECTION OF YELLOW AREA IN OVARY OF *case 2* (FIG. 2) ON 26TH DAY OF CYCLE. Note presence of thecal luteinization and absence of granulosa luteinization.

Cycle 3. No therapy, no intermenstrual bleeding, Pregnandiol was recovered from the 15th to 21st days. No specimens were available after this date.

Case 3. (Age 35 years, married, fig. 3.) Menses 11×24–29×5, the early menstrual history was characterized by considerable amount of bleeding throughout the cycle, particularly after muscular exercise. The intermenstrual bleeding is now confined to one day about the mid-cycle.

Cycle 1. No therapy, length of cycle 25 days, total pregnandiol recovered 54.5 mg. over 11 days (13–24th days of cycle).

Cycle 2. Progynon-B, 10,000 I.U. given on the 17th to 18th days, length of cycle 23 days, total pregnandiol recovered, 43 mg. over eight days (13–21st days of the cycle).

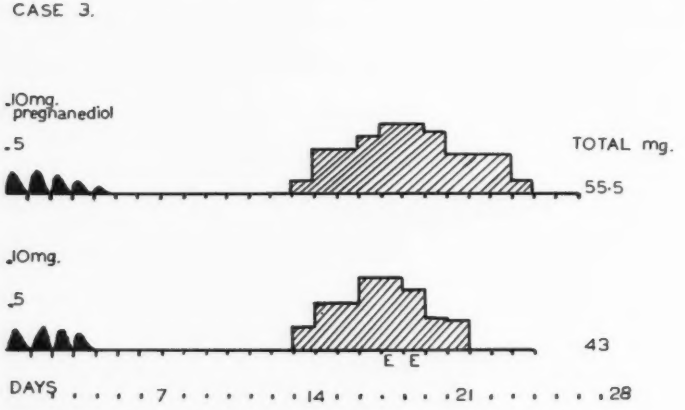

Fig. 4. Excretion of pregnandiol of a normal individual during the menstrual cycle 'E' indicates injections of 10,000 I.U. estradiol benzoate given intramuscularly.

DISCUSSION

In all cases it will be seen that the amount of pregnandiol excreted in the urine was decreased following injections of estrin in amounts varying from 20,000 to 80,000 I.U.

Case 1, cycle 4. Even though estrin injections were begun on the ninth day of the cycle, pregnandiol excretion occurred, beginning on the same day as in the 2 preceding cycles, an indication that in this patient at least 10,000 I.U. progynon-B given on the 9th, 11th, and 13th days did not prevent corpus luteum formation. The output of pregnandiol was appreciably diminished in all cycles in which estrin was administered. The length of the cycle was not affected. The dysmenorrhea was improved considerably in the cycles when estrogen was given. The dysmenorrhea was increased in severity in the cycle when pregnancy urine extract was administered. This cycle, as indicated by the graph, was delayed slightly, and the amount of pregnandiol was increased. It will be noted that pregnandiol excretion continued in this case up to the time of the onset of bleeding. No interval elapsed between cessation of pregnandiol excretion and the onset of bleeding. The presence of an interval at this time was shown by Venning and Browne (11) to be usual. However, in our experience the absence of this interval is not always accompanied by dysmenorrhea nor do all patients with dysmenorrhea show prolongation of pregnandiol excretion up to the time of onset of bleeding.

In the second case a relatively small amount of estradiol benzoate injected on the 11th, 12th, and 13th days of the cycle appears to have prevented ovulation as evidenced by the absence of pregnandiol in the urine and of a true corpus luteum in the ovary at operation. The ovaries were, however, not normal otherwise and the work of Zondek makes it probable that in normal individuals much larger doses started earlier in the cycle are needed to prevent corpus luteum formation.

The findings in these few cases confirm the work of Zondek (1) and of Kurzrok and Wilson (2). Estrin given in the luteal phase, that is after the corpus luteum has formed will depress urinary excretion of pregnandiol. The mechanism of this could theoretically be due to interference with any of the factors which may affect the excretion of this compound. Some of these are: (*a*) amount of progesterone secreted by the corpus luteum; as the corpus luteum may be under the control of the anterior pituitary the action might be one of repression of the anterior pituitary; (*b*) reduction of progesterone to pregnandiol, possibly occurring at least in part in the endometrium; (*c*) conjugation of pregnandiol with glucuronic acid; (*d*) excretion by the kidney of the sodium pregnandiol glucuronidate; (*e*) Appearance of other reduction products of progesterone. Zondek showed that if estrin were given in the luteal phase further progestational development of the endometrium was prevented. This finding would make it seem likely that the reduction in pregnandiol output is due to a decreased output of progesterone from the corpus luteum or possibly to an interference with the action of progesterone on the endometrium.

Too much emphasis should not be laid on the improvement of the symptoms of dysmenorrhea under estrin therapy during the luteal phase in *case* 1. The time of observation is too short and symptomatic improvement in dysmenorrhea too common with various forms of therapy. The significance of the marked increase in severity of symptoms in the cycle when pregnandiol excretion was increased with pregnancy urine extract is also difficult to determine. This patient[2] later was given 5 injections of 50,000 I.U. estradiol benzoate 1 every 3 days beginning on the sixth day of the cycle according to the technic of Sturgis and Albright (13) with complete relief of symptoms in 3 consecutive cycles, in 1 cycle pregnandiol was determined on the 21st and 22nd days of the cycle and none was found. The cycles were lengthened to 30 to 31 days. Wilson and Kurzrok (12) in a study of a group of cases of dysmenorrhea found a secretory endometrium in all cases. They further noted that in a large series of cases with anovulatory cycles dysmenorrhea was never present. They report that injection of 50,000 I.U. of estradiol benzoate will give relief of dysmenorrhea in 70% of cases. Sturgis and Albright (13) have found that administration of estradiol benzoate in the early part of the cycle will prevent ovulation and cause the disappearance of dysmenorrhea in that cycle. Venning and Browne (14) reported that the injection of estrin decreased the output of pregnandiol in the normal menstrual cycle. Hamblen (15) found an increased output of pregnandiol in the urine of a patient with premenstrual dermatosis and dysmenorrhea and showed that therapy with estrogenic substances would reduce the output of pregnandiol. His results are expressed as mg. of sodium pregnandiol glucuronidate of pregnandiol. The values given by Venning and Browne (11) for the normal menstrual cycle are expressed as mg. of pregnandiol. In the first cycle studied Hamblen found a total excretion during the luteal phase of 135 mg. sodium pregnandiol glucuronidate (this is equivalent to 80.5 mg. pregnandiol which is higher than the normal values 45 to 55 mg. pregnandiol given by Venning and Browne). After therapy with emmenin and estrone in 2 cycles beginning immediately post-menstrually the output was about 60 mg. sodium pregnandiol glucuronidate (36 mg. pregnandiol). In these 2 cycles the patient had no dysmenorrhea and no pre-menstrual dermatosis. That an increased output of pregnandiol is not always accompanied by dysmenorrhea or pre-menstrual dermatosis is shown by one case studied in our laboratory which excreted in 3 consecutive cycles a total of 112, 96 and 86 mg. pregnandiol (equivalent to 192, 160 and 143 mg. sodium pregnandiol glucuronidate). This patient showed no abnormal symptoms. Hamblen's results and

[2] By Dr. W. R. Foote to whom we are indebted for this information.

our own are, however, in agreement with regard to the effect of estrogens on the output of pregnandiol. It is possible that in Westman's (11) report the delay in menstruation was due to delay in occurrence of the luteal phase and that the secretory endometrium obtained at the end of 9 weeks had not persisted throughout that time but represented the effect of a corpus luteum formed shortly before the biopsy was taken, however, the number of cases in both these series is small and there may be individual variations in response. We find no evidence that estrogen in the doses used tends to prolong corpus luteum function.

SUMMARY

The excretion of pregnandiol has been studied in 3 cases which received estradiol benzoate (20,000 to 80,000 I.U.) during the menstrual cycle. There was a decreased output of pregnandiol in all cycles when estradiol benzoate was used. In one case corpus luteum formation appeared to be prevented by 10,000 I.U. given daily on the 11th, 12th and 13th days of the cycle. In another case the same dose on the 9th, 11th and 13th days failed to prevent corpus luteum formation. Injections of estradiol benzoate commenced after the luteal phase had started also decreased the output of pregnandiol in that cycle.

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CORPUS LUTEUM STUDIES

RECOVERY OF PREGNANDIOL FROM URINE

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VENNING, HENRY AND BROWNE (1–4) submitted data in 1937 and 1938 to indicate that progesterone secretion by the corpus luteum could be followed by measuring an excretion product (sodium pregnandiol glucuronidate) occurring in the urine during pregnancy or the menstrual cycle. The method for the quantitative measurement of the sodium pregnandiol glucuronidate has been reported by Venning (5, 6) and has been clinically applied by a number of investigators.

Studies made in this laboratory indicate that determinations made by the Venning method may be inaccurate as a result of partial or complete hydrolysis of the pregnandiol complex to free pregnandiol. Weil, in reporting previous work from this laboratory (7, 8) indicated that pregnandiol could be recovered quantitatively in the free form following incubation of the urine. It was thought that the recovery of free pregnandiol might offer a better index of corpus luteum function if all of the complex could be converted to the free form because the accidental loss through poor preservation would thus be obviated. The following studies were made to find out the factors influencing the hydrolysis, so that the relative merits of measuring the total pregnandiol as either (*a*) sodium pregnandiol glucuronidate or (*b*) free pregnandiol, could be determined. In carrying out this investigation each urine sample was tested for the amount of both conjugated and free pregnandiol present. It was found that neither of the two methods mentioned (Venning or Weil) was entirely reliable when used alone.

The determinations for both conjugated and free pregnandiol on the same specimen were carried out by combining the extraction procedures of Venning and Weil. This combined method was used with two variations. In one the urine was extracted fresh so that most or all of the pregnandiol recovered was in the form of sodium pregnandiol glucuronidate. In the second the urine was incubated before extraction so that most or all of the pregnandiol recovered was in the free form. Beyond this point the treatment of all urine specimens was identical. All were extracted by the combined method as outlined below.

Combined Method

The amount of sodium pregnandiol glucuronidate in the urine is determined by the method of Venning (5, 6). Free pregnandiol also is extracted quantitatively from the urine by this procedure and is carried along with the sodium pregnandiol glucuronidate until the first acetone precipitation. At this stage the sodium pregnandiol glucuronidate precipitates, and is removed by centrifugation. The acetone supernatant contains the free pregnandiol, which is removed by the method of Weil[1] slightly modified.

The clear aqueous acetone is decanted from the centrifuge cup into a 125 cc. Erlenmeyer

[1] Unpublished.

flask and allowed to stand on a steam bath until it has evaporated to dryness. Ten cc. of acetone and a few drops of 0.1 N sodium hydroxide are added and the flask warmed gently to dissolve the residue. More sodium hydroxide is introduced, a few drops at a time while the mixture is kept warm, until a total of 40 cc. has been added. It is then allowed to cool slowly and finally to stand in the icebox overnight. The free pregnandiol precipitates and is filtered out with suction. The filtrate contains the estrogens. The precipitate is washed with a small quantity (about 5 cc.) of water and a small quantity of hexane. It is transferred back to the Erlenmeyer flask with hot acetone which is allowed to evaporate off as before on the steam bath. It is then further purified by dissolving in 5 or 10 cc. of hot acetone (depending on the estimated amount of precipitate), and reprecipitating with 3 volumes of sodium hydroxide. Again after standing in the icebox overnight it is filtered off, washed with water and transferred to a 50 cc. Erlenmeyer flask. A third precipitation from 2, 3 or 5 cc. of ethyl alcohol by the addition of two volumes of water should complete the purification. Occasionally this last step must be repeated. In carrying out the above precipitations care must be taken to introduce the sodium hydroxide (or water) very gradually, while the mixture is being warmed gently on the steam bath. If the aqueous portion is added too rapidly a fine suspension of pregnandiol crystals, very difficult to separate from the supernatant, will result. The final precipitate is filtered off, transferred with ethyl alcohol to a small flask and weighed after the alcohol has been evaporated off in a drying oven.[2]

Efficiency of the technic of extraction. The efficiency of the technic for the quantitative recovery of sodium pregnandiol glucuronidate has been carefully worked out by Venning. A check on the accuracy of the method outlined above in the recovery of free pregnandiol, however, may be had by comparing the amount of pregnandiol obtained from the extraction of fresh urine (extracted as the glucuronidate and calculated in terms of free) with the amount of pregnandiol obtained from an equal portion of the same urine extracted after incubation. The results of such an experiment are shown in table 1. It will be seen from comparison of the results that the totals by the two methods (the combined method for fresh urine and the combined method for incubated urine) are generally consistent, indicating that the technic outlined for the recovery of free pregnandiol gives results roughly comparable to the Venning technic.

TABLE 1. COMPARISON OF THE TOTAL YIELD OF PREGNANDIOL FROM EQUAL FRACTIONS OF THE SAME SPECIMEN OF PREGNANCY URINE RECOVERED BY TWO DIFFERENT PROCEDURES

Sample	Extracted fresh or incubated	Pregnandiol		Total pregnandiol calculated as free
		Conjugated	Free	
		mg.	*mg.*	*mg.*
60*a*	Fresh	25.4	1.1[1]	16.3
60*b*	Incubated	0	15.0	15.0
60*c*	Incubated	0	14.8	14.8

[1] A small quantity of conjugated pregnandiol was hydrolyzed during the collection period. The technic of extraction was the same in all cases; each lot was examined for both sodium pregnandiol glucuronidate and free pregnandiol by the combined method outlined above. The volumes of the lots were all equal.

Conditions Influencing the Hydrolysis of the Pregnandiol Complex

The following studies were made on the recovery of sodium pregnandiol glucuronidate to find out where and under what conditions hydrolysis may occur.

(*a*) *Routine collection with preservative.* Studies were first made on urine from both pregnancy and normal menstrual cycles to determine whether collection with a

[2] The melting point of this crude pregnandiol which is weighed ranges from 224–232°. Recrystallization of this from alcohol gives a pure product melting at 238°.

preservative (6 drops of tricresol per 24-hour specimen) and extraction within 1 to 6 hours from the end of collection would prevent breakdown and give accurate results if the sodium pregnandiol glucuronidate alone were measured. These collections were made during the summer months. Refrigeration was not used during collection because it was considered impractical in any method designed for clinical purposes. The specimens were extracted as soon as they arrived in the laboratory, in two instances within one hour after the end of the collection period. The results are shown in table 2.

TABLE 2. EXTRACTION OF BOTH CONJUGATED AND FREE PREGNANDIOL FROM THE SAME SPECIMEN OF URINE, COLLECTED WITH PRESERVATIVE

Specimen No.	Amount	Type	Period of Collection	No. of hr. from end of collection period to time of extraction	Pregnandiol Conjugated	Pregnandiol Free	Total pregnandiol calculated as free	% of total pregnandiol recovered as free
	cc.		hr.		mg.	mg.	mg.	
66	800	PU	24	1½	20.0	0.7	12.7	5½
77	2160	PU	24[1]	1	99.3	22.5	82.1	27½
78	1650	PU	24[1]	1	109.0	2.3	67.7	3
840[2]	3860	MC	48	not more than 6	0	1.9	1.9	100
841[2]	4800	MC	48	not more than 6	6.4	1.6	5.4	29½
843[2]	2800	MC	48	not more than 6	5.9	2.9	6.4	45

PU Pregnancy urine.
MC Urine from the menstrual cycle.
[1] Twenty-four-hour specimens from 2 patients collected simultaneously, then combined and extracted as one specimen.
[2] Three consecutive 48-hour determinations (840-841-843) on the same patient.

All specimens showed the presence of some free pregnandiol even when collected in the presence of tricresol and extracted one hour after the end of the collection period. The amount of free pregnandiol liberated may vary from a negligible quantity up to 100 per cent. The hydrolysis occurs in urine both of pregnancy and the menstrual cycle. In urine of the menstrual cycle such hydrolysis is of considerable importance if measurement of sodium pregnandiol glucuronidate alone is relied upon. The total pregnandiol excretion may be so small that the breakdown of a few milligrams to the free form, represents a large percentage of the total.

(*b*) *Effects of boiling the urine.* It was decided to boil the urine and see whether this would by destruction of any enzymes present, prevent further hydrolysis. The results are shown in table 3.

TABLE 3. RECOVERY OF CONJUGATED AND FREE PREGNANDIOL FROM EQUAL LOTS OF THE SAME BATCH OF URINE AFTER BOILING AND INCUBATION (*A*, CONTROL BEFORE BOILING AND INCUBATION; *B* AND *C*, AFTER BOILING AND INCUBATION; *D* AND *E*, INCUBATED WITHOUT BOILING)

Sample	Amount	Boiled or not boiled	Incubation period at 37°	pH	Recovery Combined	Recovery Free
	cc.		hr.		mg.	mg.
34*A*[1]	700	Not boiled	Not incubated	—	13.1	9.1
34*B*	700	Boiled and kept sterile	24	6	13.0	8.4
34*C*	700	Boiled and kept sterile	24	7	13.0	7.2
34*D*	700	Not boiled	24	6	1.3	14.4
34*E*	700	Not boiled	24	7	1.3	17.6

[1] Lot *A* is the non-incubated control. Partial hydrolysis had already occurred before the experiment was started.

A number of specimens of pregnancy urine were pooled and divided into equal lots as indicated. That some hydrolysis had already occurred before the experiment was started is evident from the control (*34a*) which was extracted without incubation. Two lots (*34b* and *c*) were heated to boiling for 5 minutes, cooled and kept sterile. Twenty-four-hour incubation at 37° failed to produce any additional free pregnandiol. Two other lots (*34d* and *e*) of the same specimen, not boiled, but incubated under conditions otherwise identical showed nearly complete conversion of conjugated to free pregnandiol at the end of the same period. The failure of incubation to liberate free pregnandiol in boiled sterile urine, although it may readily be liberated in non-boiled urine by the same means, indicates that enzyme action is essential to the process.

(*c*) *Collection by catheterization.* Since free pregnandiol occurs in urine collected in the presence of preservative, and since breakdown of the complex to the free form is stopped by boiling, an attempt was made to determine whether or not this breakdown was due to an endogenous enzyme excreted in the urine. If so, free pregnandiol might be liberated in the bladder before the urine is voided, because temperature and PH conditions in the bladder in general coincide with those under which hydrolysis can occur in vitro. Specimens of urine from the various months of pregnancy were obtained by catheterization, pooled and extracted for free and conjugated pregnandiol.

TABLE 4. EXTRACTION OF BOTH CONJUGATED AND FREE PREGNANDIOL FROM THE SAME BATCH OF CATHETERIZED URINE[1]

Specimen No.	No. of patients	Amount	Incubation at 37° and PH 6 to 7.5	Pregnandiol Conjugated	Pregnandiol Free
		cc.	*hr.*	*mg.*	*mg.*
43	1	465	27	11.0	0
44	3	520	40	13.0	0
79	3	210	48	14.8	0
80	16	3468	not incubated	249.2	2

[1] The various urine samples contained in this batch were extracted immediately after catheterization. Extraction was never delayed longer than 15 minutes. The butyl alcohol extracts were pooled and run through as one specimen.

Table 4 shows the results. Several groups of specimens were kept sterile and incubated under conditions which had previously been shown to be the optimum for liberation of free pregnandiol in urine collected in the ordinary way (43, 44, 79). No free pregnandiol was recovered.

A second group of specimens (80) was obtained over a period of time by catheterizing 16 patients just before delivery. These specimens were extracted immediately and the butyl alcohol extracts pooled until a total representing 3469 cc. of urine has been obtained. The results showed a negligible amount (only 2 mg.) of free pregnandiol in comparison with 249 mg. of sodium pregnandiol glucuronidate. It was concluded that an endogenous enzyme is not present and that hydrolysis of the pregnandiol complex does not normally occur in the bladder.

Since this is the case a suitable preservative might be found which could prevent hydrolysis and make the determination of sodium pregnandiol glucuronidate alone a reliable index of measurement in routine work.

Venning (6) has found that 0.5% of sodium cyanide will prevent hydrolysis for a period of 4 days if the temperature does not rise above 20°. In the authors' opinion, however, this substance is too dangerous for use in routine clinical studies.

(*d*) *Incubation of urine.* The possibility of recovering all of the pregnandiol in the free form following incubation of the urine was investigated as a means of

avoiding the loss through partial hydrolysis encountered in the measurement of the conjugated form discussed above.

Since the splitting of sodium pregnandiol glucuronidate occurs in voided, but not in sterile catheterized urine, the enzymatic action is apparently associated with bacterial growth. Investigations were undertaken to find out what physical factors would be favorable to such action, and whether or not the presence of the necessary enzymatic factors could always be relied on, in urine of pregnancy and the menstrual cycle.

Large batches of urine were divided into equal lots and incubated under varied conditions of time, pH and temperature. It was found that incubation for less than 24 hours at 38° often led to incomplete hydrolysis, while incubation for a longer time tended to increase the impurities in the yield. A fairly wide pH range seemed satisfactory: hydrolysis occurred readily between pH 6 and 8, was not much hampered by an alkalinity as high as pH 9, but was considerably reduced by acidity as low as pH 2–3. Hydrolysis occurred on incubation for 25 hours at a temperature of 50°.

In spite of these non-specific conditions under which hydrolysis seems to take place, in some instances the reaction will go only part way to completion and a considerable portion of the sodium pregnandiol glucuronidate remains unhydrolyzed (as high as 80% in several instances).

Apparently the important factor is the presence of the proper enzyme in sufficient quantity and the results just cited indicate that this cannot always be relied upon. No urine from female patients was found, however, in which at least a partial hydrolysis did not take place under the conditions of incubation mentioned. Different specimens from the same patient may vary in the degree of hydrolysis of sodium pregnandiol glucuronidate which will take place on incubation under the conditions described above. Lots taken from the same specimen or the same pooled collection of urine seem to behave consistently with respect to the amount of hydrolysis which will take place, even at different pH levels.

Recovery of Sodium Pregnandiol Glucuronidate Added to Urine of Male Patients and Recovered as Free Pregnandiol

A number of experimental samples of pregnancy urine used in these determinations were composed of specimens obtained simultaneously from a number of patients and pooled. As the hydrolysis never failed to take place in such specimens it was thought that the active factor must be readily transferable from one specimen to another and that it was closely associated with bacterial growth in the urine.

Further evidence for this view was obtained from studies on urine from male patients to which known amounts of sodium pregnandiol glucuronidate had been added. One group of these specimens was incubated under conditions previously noted to be favorable to the liberation of free pregnandiol. There were 4 separate batches of pooled urine in this group, each being subdivided into 4 lots. Hydrolysis occurred in only 4 lots, all from the same batch. No free pregnandiol appeared in any of the 12 lots of the 3 remaining batches. The enzyme which causes breakdown is thus not necessarily limited to female patients but may occur in the urine of male patients as well. Since the bacterial content of urine from male patients is usually less than that of urine from female patients it was to be expected that many specimens would occur in which there would be no splitting of the pregnandiol complex.

A second group of specimens from male patients containing added sodium pregnandiol glucurondiate was boiled to insure destruction of the enzyme and then divided into different lots and incubated. Some were kept sterile and others inoculated with 10 cc. each of various samples of pregnancy urine, known from previous determina-

tions to contain the factors producing hydrolysis. No hydrolysis occurred in the specimens which were kept sterile. Nearly complete or complete liberation of free pregnandiol occurred in the inoculated specimens. This seems to indicate that a bacterial enzyme is responsible for the splitting.[3]

The above data indicate that incubation of the urine does not insure hydrolysis since the presence of the proper bacteria cannot always be relied upon. Veitch (10) has reported hydrolysis of sodium pregnandiol glucuronidate directly in the urine with the enzyme emulsin, but this work is still incomplete.

SUMMARY

Rôle of bacteria in hydrolysis of the pregnandiol complex. It seems probable that the liberation of free pregnandiol from sodium pregnandiol glucuronidate is brought about by the action of a bacterial enzyme. This asumption is based upon the following observations.

(*a*) The hydrolysis will not take place in boiled sterile urine or in sterile catheterized urine even when incubated.

(*b*) The enzyme capable of causing hydrolysis appeared in all of the urine from female patients studied. In the specimens investigated, either from pregnancy or the menstrual cycle at least a partial hydrolysis could be produced by incubation under the conditions outlined. In some instances hydrolysis was observed to go only part way to completion during the time allotted. If a bacterial enzyme were the cause of this action, the cessation of activity might be explained on the basis of overgrowth by another organism.

(*c*) The enzyme occurs naturally in some specimens from male patients but not very many. This might be explained on the basis of the known low bacterial content of such urine.

(*d*) The enzyme can be transferred from a specimen in which it is active to a sterile sample of urine from male patients to which sodium pregnandiol glucuronidate has been added, and, on incubation under proper conditions will bring about liberation of the free pregnandiol. This behavior apparently indicates that bacterial action is the basic factor in the process of hydrolysis.

Evaluation of methods. An attempt has been made to evaluate two methods for the determination of pregnandiol excretion in the urine, by means of a combined method with which it is possible to recover both forms of pregnandiol from the same specimen of urine.

The chief objection to determinations based only upon recovery of sodium pregnandiol glucuronidate is the difficulty of preventing hydrolysis during collection of the urine. The chief objection to determinations based only upon the recovery of free pregnandiol is the occasional failure to convert all of the pregnandiol to the free form by incubation.

The method based upon the determination of sodium pregnandiol glucuronidate has the advantage of a much heavier molecule which is an important factor in attaining accuracy since the yields are measured gravimetrically.

The method based upon the recovery of free pregnandiol may have an advantage in the investigation of conditions where there is an infection in the urinary tract and where some hydrolysis may occur before the urine is voided.

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[3] An unsuccessful attempt to obtain splitting of the pregnandiol complex in boiled pregnancy urine by the addition of pure cultures of several types of bacteria was made. Cultures of B. Coli-G-1, B. Coli-G-2 and B. Paratyphosum E-12 were used(9). The amount of hydrolysis obtained was so slight that no conclusions can be drawn, though more free pregnandiol was recovered from the inoculated specimens than from the control. Possibly the amount of culture introduced was insufficient for the incubation period allotted.

Where a reliable measure of pregnandiol excretion is desired (especially in hot weather) it seems best to supplement Venning's procedure with a test for free pregnandiol as outlined in the combined method described above.

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ALTERATION OF URINARY EXCRETION OF ANDROGENS BY ESTROGENIC THERAPY[1,2]

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SOME MEMBERS of our group (1) have reported previously that urinary titers of androgens are decreased by injections of estrogens. The present communication is concerned with an extension of these studies to include a larger number of patients who were treated with diverse estrogens, administered orally and by injection.

METHOD

Urine was hydrolyzed and extracted prior to quantitation by a method described by Cuyler and Baptist (2). The colorimetric method of Oesting (3) was employed for

TABLE 1. PATIENTS WITH SUBJECTIVE SYMPTOMATOLOGY ASSOCIATED WITH CLIMACTERIC

Patient	Age	Average androgenic titers before treatment	Estrogenic treatment: Average dosage (daily or as noted)	Estrogenic treatment: Total estrogens given during period of study	Average androgenic titers during treatment	Alteration in androgens due to treatment
		I.U.			I.U.	%
I	39	68	Es 720 du	15,120 du	48	−29
II	50	135	Es 900 du	38,700 du	25	−82
III	57	99	Es 1800 du	23,400 du	73	−26
IV	42	101	Es 720 du	12,120 du	95	− 6
V	49	86	Es 300 du	41,180 du	92	+ 3
			Es 450 du	8,550 du	84	− 6
VI	35	78	Es 465 du	32,550 du	65	−17
VII	44	97	Es 465 du	55,320 du	88	− 9
VIII	50	84	Es 900 du	14,440 du	41	−51
			Es 400 du	74,880 du	29	−65
IX	48	76	Ed B 0.33 mg. (Thrice weekly)	4.3 mg.	55	−28
		32	Es 360 du	9,360 du	33	+ 3
X	47	64	Ed B 0.33 mg. (Twice weekly)	2.3 mg.	50	−22
Average for group	46	84			60	−26

Code to abbreviations: Es=estriol glucuronide
Ed B=estradiol benzoate
du=day oral units

[1] Read before the 24th annual meeting of the Association for the Study of Internal Secretions, New York City, June 1940.

[2] Aided by a research grant from the Ciba Pharmaceutical Products Incorporated, Summit, New Jersey.

[3] Ciba Fellow in Clinical Endocrinology.

the estimation of total androgenic titers. Consecutive 24-hour specimens of urine were examined prior to initiation of therapy for intervals which ranged from 9 to 28 days in order to establish each patient's average daily excretion of androgens during the pretreatment period. During one or more series of therapy, daily 24-hour specimens of each patient were titrated for intervals which varied from 5 to 26 days. Only treatment which was given during the periods when hormonal titrations were being made are reported. No correlational studies by methods of bioassay were made. The results, although reported in international units, do not warrant inferences as to the biologic activity or the qualitative identity of the 'androgens' determined.

RESULTS

The studies comprise 835 determinations of androgenic titers upon a total of 22 women. These women were divided into 3 groups, depending upon their gynecologic and endocrine status.

TABLE 2. PATIENTS WITH MENSTRUAL IRREGULARITIES RELATED TO HYPOOVARIANISM

Patient	Age	Average androgenic titers before treatment	Estrogenic treatment: Average dosage (daily or as noted)	Estrogenic treatment: Total estrogens given during period of study	Average androgenic titers during treatment	Alteration in androgens due to treatment
		I.U.			I.U.	%
XI	18	33	Ed D 1 mg. (Thrice weekly)	6 mg.	29	−12
XII	32	78	Ed B 0.33 mg. (Thrice weekly)	1.9 mg.	59	−24
			Ed B 0.33 mg. (Thrice weekly)	2.6 mg.	53	−32
			Es 1800 du	30,600 du	66	−16
XIII	29	26	Ed B 0.33 mg. (Thrice weekly)	5.6 mg.	12	−54
			Ed B 0.33 mg. (Thrice weekly)	1.6 mg.	10	−62
			Ed 2 mg. Es 3900 du	21 mg. 39,000 du	15	−42
			Ed 2 mg. Es 3900 du	26.4 mg. 42,900 du	22	−15
			Ed B 0.33 mg. Es 3900 du	1.9 mg. 7650 du	18	−31
XIV	30	69	Ed B 0.33 mg.	5.6 mg.	48	−33
			Ed B 0.33 mg.	5.6 mg.	61	−12
			Fd B 0.66 mg.	3.5 mg.	42	−39
		41	Ed B 0.33 mg. Es 1800 du	5.2 mg. 31,600 du	30	−27
XV	16	145	Ed D 2.5 mg. (Thrice weekly)	57.5 mg.	101	−30
XVI	26	101	Ed 2 mg.	28 mg.	62	−39
XVII	17	63	Ed 2 mg.	28 mg.	58	− 8
Average for group	24	70			43	−30

Code to abbreviations: Ed D = estradiol dipropionate
Ed = estradiol

Group 1. This consists of 10 women who were investigated and treated because of subjective symptomatology associated with the climacteric. Their average age was 46 years. The results in detail are summarized in table 1.

The treatment of these patients was as follows: 8 received estriol glucuronide,[4]

[4] Emmenin, supplied by Ayerst, McKenna & Harrison, Montreal, Canada.

TABLE 3. PATIENTS WITH SUBJECTIVE SYMPTOMATOLOGY ASSOCIATED WITH CYCLIC MENSES

Patient	Age	Average androgenic titers before treatment	Estrogenic therapy: Average dosage (daily or as noted)	Estrogenic therapy: Total estrogens given during period of study	Average androgenic titers during treatment	Alteration in androgens due to treatment
		I.U.			I.U.	%
XVIII	28	75	Es 180 du	3420 du	58	−23
XIX	42	45	Es 240 du	4320 du	31	−31
XX	22	64	Es 1800 du	6300 du	55	−14
XXI	21	55	Ed B 0.33 mg. (Thrice weekly)	0.99 mg.	58	+ 5
			Ed B 0.66 mg. (Thrice weekly)	1.32 mg.	39	−29
XXII	23	55	Ed B 0.33 mg. (Thrice weekly)	1.98 mg.	60	+ 8
Average for group	27	59			50	−14

given orally (average daily dose 680 day oral units); 1 received estradiol benzoate[5] by injection (0.333 mg. thrice weekly); and 1 was given both estriol glucuronide and estradiol benzoate.

The average daily androgenic titer for the group prior to therapy was 84 I.U. The average titer for the group during treatment was 60 I.U. (Determinations of androgenic titers were begun on the average of 44 days after initiation of therapy

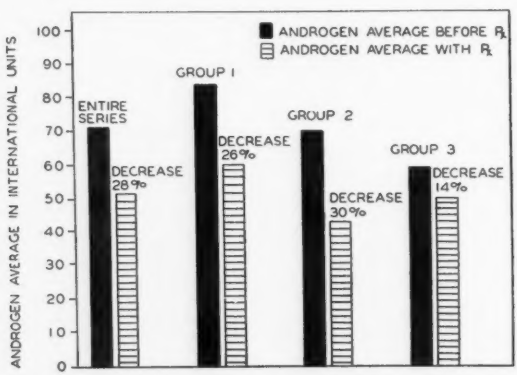

Fig. 1. THE ALTERATIONS PRODUCED IN ANDROGENIC TITERS OF PATIENTS IN GROUPS 1, 2 AND 3 BY ESTROGENIC THERAPY.

with estriol glucuronide.) The reduction of androgenic titers averaged 26% for the group. All patients experienced symptomatic improvement during treatment.

Group 2. This consists of 7 women who were investigated and treated because of menstrual irregularities related to hypoovarianism. Their average age was 24 years. The results in detail are summarized in table 2.

Various estrogens used included estradiol,[6] estradiol benzoate, estradiol dipro-

[5] Progynon-B, supplied by Schering Corporation, Bloomfield, New Jersey; Ben-oveylin, supplied by Ciba Pharmaceutical Products Incorporated, Summit, New Jersey.

[6] Progynon-DH, supplied by Schering Corporation, Bloomfield, New Jersey.

pionate[7] and estriol glucuronide. Average doses were not calculable due to marked individual variations (table 2).

The average daily androgenic titer for the group prior to therapy was 70 I.U. The average titer for the group during treatment was 43 I.U. The reduction of androgenic titers averaged 30% for the group. Clinical responses of the patients varied from negligible ones to satisfactory improvements.

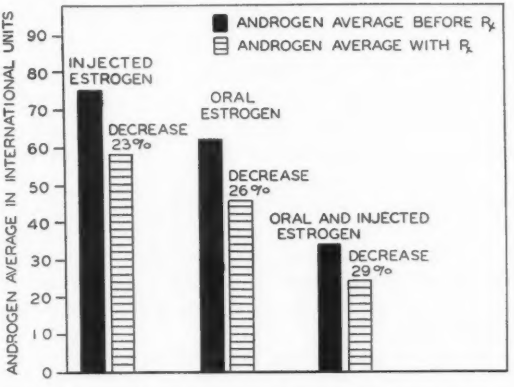

Fig. 2. THE COMPARISON OF THE EFFECTS OF INJECTED, ORAL AND INJECTED AND ORAL ESTROGENIC THERAPY ON ANDROGENIC TITERS

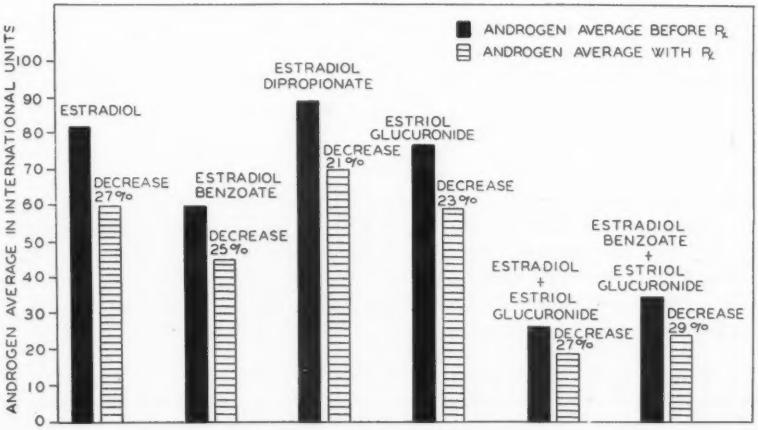

Fig. 3. THE ALTERATIONS IN ANDROGENIC TITERS AFFECTED BY INDIVIDUAL ESTROGENS.

Group 3. This consists of 5 women who were investigated and treated because of various symptoms associated with cyclic uterine bleeding. Their average age was 27 years. The results in detail are summarized in table 3.

The treatment of these patients was as follows: 3 received estriol glucuronide (average daily dose 740 day oral units); and 2 were given estradiol benzoate (0.33 or 0.666 mg. every second day).

[7] Di-ovocylin, supplied by Ciba Pharmaceutical Products Incorporated, Summit, New Jersey.

The average daily androgenic titer for the group prior to therapy was 59 I.U. The average titer for the group during treatment was 50 I.U. The reduction of androgenic titers averaged 14% for the group. All patients experienced symptomatic improvement during therapy.

The effects of estrogenic therapy upon the average titers of the patients comprising these 3 groups is presented graphically in figure 1. The relative efficiency of orally administered and injected estrogens in lowering androgenic titers is illustrated in figure 2. The effects of individual estrogens upon androgenic titers are shown in figure 3.

DISCUSSION

The average daily androgenic titers for each of the 3 groups of patients studied were elevated definitely as compared with an average of 34 I.U. established for a carefully investigated group of 13 healthy and cyclic young women (4). The effects of estrogenic therapy upon the androgenic titers of healthy women and of those excessively elevated titers of women with adrenogenitalism have not been investigated. It is urged that these studies do not warrant the assumption that estrogenic therapy can negate the virilizing effects of excessive androgenic secretion associated with hyperplasia or neoplasm of the adrenal cortex. These studies do support the belief that an androgen/estrogen balance exists which is altered by changes in the concentration of the component elements.

In no instances were the androgenic titers reduced lower than average normal levels. It would be interesting to study androgenic titers in patients with hyperestrogenism (due to granulosa cell tumor of the ovaries) or patients who were receiving massive doses of estrogens. In these instances androgenic titers may be found reduced to subnormal levels.

These studies have not been concerned with an assessment of the therapeutic or pharmacologic activity of the individual estrogens employed. The statement is warranted, however, that orally administered estrogens are as effective in reducing moderately elevated androgenic titers as injected estrogens, even though the former are used in smaller amounts than the latter.

SUMMARY

The androgenic titers of 22 women, who had various grades of ovarian failure, were quantitated by Oesting's method prior to and during oral and injectional therapy with various estrogens. The percentages of decrease during therapy of these moderately elevated titers were 26, 30 and 14 for the 3 groups of patients studied. Orally administered estrogens, although given in smaller quantities than injected estrogens, were equally as effective in altering androgenic values.

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ORAL ADMINISTRATION OF DIETHYL STILBESTROL DIPROPIONATE[1]

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THE INTRODUCTION of diethyl stilbestrol by Dodds and coworkers in 1938 (1) initiated an intensive investigation of synthetic estrogenic substances. A large number of reports dealing with clinical (2–22) and laboratory studies (23–33) of this and related compounds have subsequently appeared. The efficacy of this group of compounds as potent estrogens has been adequately demonstrated, both experimentally and clinically. However, toxic manifestations tending to limit the clinical application of such drugs have been generally noted (27, 34–38). The toxicity of stilbestrol led to the utilization of various derivatives in an effort to retain the estrogenic potency of the drug and eradicate its toxic effects. Of the many esters studied, diethyl stilbestrol dipropionate has yielded the least toxic manifestations, while maintaining the same degree of estrogenic potency (38).

Our experiences with the synthetic estrogen, stilbestrol, administered both intramuscularly and orally, were very satisfactory in the control of syndromes occasioned by estrogenic insufficiency (39, 40), despite the occurrence of toxic manifestations, nausea and less frequently vomiting. Others have reported considerable variation in unpleasant effects, but all authors are in accord as to the estrogenic potency of the drug. Bishop, et al., have reported their experiences with the dipropionate ester of stilbestrol indicating that its estrogenic potency is equal to that of stilbestrol, and that the toxic manifestations are less (38). In their series, 21.6% of cases reported nausea and/or vomiting in contrast to the universal report of toxic manifestations occurring in the majority of cases treated with stilbestrol.

The present report includes 92 cases to whom the dipropionate ester of stilbestrol[2] was administered orally in the menopausal syndrome, both physiological and artificial, senile vaginitis and hypoestrinism.

Group 1. The physiological menopausal syndrome group consisted of 51 cases, ranging in age from 36 to 58, exhibiting subjective symptoms varying in combination and degree. The symptomatology included dizziness, hot flushes, headaches, insomnia, nervousness, irritability, muscle and joint pain, nausea, lassitude, fatigue, depression, and frigidity. Patients with organic disease to which such symptoms might be attributed have been eliminated.

The therapeutic regime instituted in this series consisted of 1 mg. of diethyl stilbestrol dipropionate orally 3 times daily. After one week's treatment the response the patient to the medication was ascertained, and the dosage was then varied in accordance with the individual's reaction. When symptoms were mild or markedly im-

[1] Aided by a grant from the Anonymous Research Fund.

[2] A generous supply of this drug was furnished for this study by the Winthrop Chemical Company.

proved, the daily dose was reduced to 0.3 mg., whereas in those cases showing very little or no response, the daily dosage was increased to as much as 15 mg. Individualization of therapy was necessary.

Of the 51 cases, 25 were relieved of all symptoms, 16 were improved, 3 showed only slight improvement, and 7 exhibited no response; of the latter, one was intolerant to the drug.

Group 2. The group of patients exhibiting menopausal symptoms initiated by surgical or roentgenological procedures consisted of 35 cases. These patients ranged in age from 25 to 49.

The fundamental method of treatment in this group was that employed in group 1, and as before, individualization of therapy was followed. In general, a large maintenance dose was necessary for relief of this group.

Of the 35 cases, 19 were completely relieved, 13 were improved, while 2 were slightly improved, and 1 evidenced no improvement.

Group 3. There were 6 young women exhibiting evidence of hypoestrinism as manifested by menstrual irregularities and symptoms suggestive of premature menopause. These symptoms included headaches, nervousness, irritability, occasional hot flushes, anxiety, depression and diminished libido.

In this group of cases more so than in the others, individualization of therapy was essential. Since most of the complaints were of a cyclic nature, usually preceding or associated with the menstrual flow, the use of the drug was restricted to that time. Of the 6 cases in this group, all were improved.

Group 4. Atrophic changes of the genitalia were found in 8 cases, of which 3 followed artificial menopause. Four were associated with the physiological menopausal syndrome, and 1 exhibited senile vaginitis without concomitant menopausal symptoms. Here again individualization of therapy was instituted. In all cases, keratinization of the vaginal epithelium followed the oral administration of the drug. This change was noted within as short an interval as one week, and with as little as 7 mg., whereas usually 3 weeks elapsed and 21 mg. were administered before complete relief was obtained. All patients were completely relieved, but recurrences were frequent 6 to 12 weeks after discontinuance of therapy.

Whereas senile vaginitis can be controlled by diethyl stilbestrol dipropionate orally, and keratinization of the vaginal epithelium can be demonstrated, the associated atrophic changes do not show the same degree of regression that follows the vaginal administration of biologic estrogens. In our experience, the administration of stilbestrol dipropionate in suppository form, has been unsatisfactory in the control of any form of estrogenic insufficiency.

Toxicity. The only toxic manifestations evidenced in this series were nausea, and to a lesser extent, vomiting. These were noted in 15%. We have not observed such toxic changes as exfoliative dermatitis (41), diarrhea, skin rashes, psychosis, vertigo, severe thirst, paresthesia (16), or toxic jaundice (36), as have been noted by other authors. Only one patient could not tolerate the drug in any dosage.

The possible tumorogenic and carcinogenic activity of estrogenic substances has been widely debated in the literature. While this property has been demonstrated in the experimental animal (42–45) Novak (46) has indicated that there is no clinical evidence to deter utilization of estrogens to alleviate deficiency syndromes in man, an opinion which is shared by Zondek (22).

DISCUSSION

The increasing popularity of estrogenic substances both biologic and synthetic, for the relief of deficiency syndromes is well founded. While no ideal substance has

been discovered the oral administration of such drugs is preferable to other routes. The synthetic estrogens have been adequately proven to be potent when so administered, and justify cautious clinical trial. It is to be hoped that further modification of the synthetic estrogens will eradicate toxic manifestations produced by the drugs in current use. However, many patients volunteer the information that the mild nausea that they experience is preferable to the repeated hypodermic administration of biologic estrogens.

Individualization of therapy is necessary! We have noted extreme variation in the dosage required to alleviate symptoms. As little as 4.2 mg. has relieved cases of mild hypoestrinism, and, as much as 315 mg. given over a period of six weeks was required before relief of symptoms was obtained in a case of artificial menopause in a young woman. It is interesting to note that this case received a total of 510 mg. over a period of 9 months without ill effect. In fact the patient was able to promptly detect substitution of a placebo for the drug. This dosage represents 1,275,000 I.U. of estrogenic substance!

In addition to the relief of such symptoms as headaches, hot flushes and dizziness, many patients also reported a sense of well being, and relief of anxiety, equal to that previously obtained with the use of biologic estrogens in these types of cases (47).

Recurrences have been observed following cessation of therapy in periods varying from 6 to 12 weeks. Resumption of treatment in every such instance was followed by complete relief. Small maintenance doses thereafter, gradually diminishing in size have been found to prevent such recurrences of symptoms.

A unique observation on this group of patients was the reported occurrence of an increased frequency of urination without dysuria. None of these patients had previously manifested symptoms of cystitis, nor did they develop them. We are at a loss to explain the mechanism of this frequency. However, since it is well established that stilbestrol as well as the biologic estrogens, exert a marked vasodilator effect we may account for this frequency as resulting from diuresis induced by the increased blood flow through the kidney. However, since stilbene derivatives are excreted in the urine, the frequency observed may be occasioned by a diuresis either compulsatory in character or induced by kidney irritation. Further investigation of this phenomenon is necessary.

SUMMARY

Diethyl stilbestrol dipropionate is a potent synthetic estrogen which can be efficiently administered orally with less toxic manifestations than stilbestrol.

In our series of 51 cases of physiological menopausal syndrome 25 were relieved, 16 were improved, and 3 were slightly improved, while 7 exhibited no response. In our series of 35 cases of artificial menopausal syndrome 9 were relieved, 13 were improved, and 2 were slightly improved, and 1 showed no improvement. In 6 cases of hypoestrinism, all were relieved. Relief was obtained in all of our 8 cases of senile vaginitis, but recurrence of this complaint frequently followed cessation of therapy. Only one patient was completely intolerant to the drug.

Emphasis is placed upon individualization of therapy.

We wish to thank Drs. Oran Prejean, J. D. Talbot, and E. W. Nelson for their assistance in compiling this series.

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CASTRATION IN THE MALE

NOTES ON THE HYPOTHALAMICO-PITUITARY-GONADAL SYSTEM

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RECENTLY Feinier and Rothman (1) stated that "It appears that in the full-grown adult the only indispensable sexual function of the testes is that of procreation and that libido and potency are functions of and controlled by the prepituitary and psychic centers." Their case report was of an adult male castrate who "retained libido and prowess for thirty years after complete orchidectomy."

The present paper is a report of two adult castrates who lost libido and potency, one of whom was treated by male sex hormone (testosterone propionate).

Case 1. H. K., aged 46, divorced, white male, was seen November 4, 1937 because of impotency and multiple arthritis. In 1923 a left orchidectomy had been performed for tuberculosis of the epididymis and testicle. Within one month after the operation the hair of the scalp came out in patches (alopecia areata). Libido and potency were not affected.

One year later (1924) the right epididymis and testicle were involved by tuberculosis necessitating the removal of all but a small portion of the testicle.

He had been in good health previous to the gonadal tuberculosis. His father died of carcinoma of the rectum and his mother died of carcinoma of the breast.

He was the father of a 17-year-old boy and was divorced in 1928, 4 years after the second operation.

For two years after the last operation, in 1924, he retained both libido and potency. During this time the hair on his scalp kept coming out in patches but would come in again. The necessity of shaving decreased from a daily task to 3 times a week. At the end of 2 years following the second operation, the hair on his scalp had disappeared entirely so that he was totally bald. The eyebrows disappeared, the beard became so scant that he shaved but once a week and even that was not, as he said, really necessary. The axillary, truncal and pubic hair were gradually lost. He gained 35 lb. in weight over a period of 13 years. Arthritis of several joints became troublesome 2 years after the second operation and walking produced pain in the right large toe.

He had a period of hot flashes, and at this time (2 years after the second orchidectomy) he lost all interest in women. As he said "I don't care to touch a woman and they don't interest me." His wife divorced him in 1928, 4 years after castration, and 2 years after his loss of libido and potency. He stated that the latter was not the cause of the divorce, but gave no reason why one was granted.

The impotency and loss of libido had been present for 13 years previous to his being seen in November, 1937.

Physical examination. This showed a well nourished male with an almost complete alopecia, a few sparse facial hairs were present and the eyebrows, axillary, truncal and pubic hairs were absent. The scalp on the vertex showed no hair growth, and but a few grey hairs were present in the temporo-parietal-occipital regions.

The skin of the body was smooth, and the breasts were suggestive of the feminine type. There was a small mass of firm, hard, nodular tissue present in the location of the right testicle (fig. 1).

No objective evidence of arthritis was seen except in the right large toe. This was definitely enlarged at the tarsometatarsal joint.

The blood pressure was systolic 128, diastolic 82. Blood nitrogen and blood sugar were normal. Blood Kahn test result was negative. The urine on microscopic examination showed several pus cells. The blood count showed a mild secondary anemia. The B.M.R. rate was −10%.

X-ray of the right large toe was reported as showing "A large spur formation on the dorsal aspect of the distal end of the first metatarsal. There is some diminution of the joint space. There is also a spur formation shown on the upper end of the proximal phalanx of the great toe."

Unfortunately for our purposes, to show the contrast previous and subsequent to therapy, he refused to have his photograph taken. He also was not willing to take any form of treatment which would restore his libido and potency. He said, "Why should I bother with women? I'm satisfied; they don't interest me." Only by persuasion that this would benefit his arthritis did he finally consent to treatment.

Beginning November 20, 1937, 10 mg. of testosterone propionate was given 3 times a week until a total of 120 mg. had been given.[1] This small dosage had no influence either physically or psychically. This dosage was given before the larger doses of 25 mg. were available. He was then given 2 cc. of a gonadotropic factor (500 U per cc.) in the hope of stimulating any testicular cells that might be present in the remnant. A total of 15,000 U was given over a period of 2 months with no apparent benefit. He was not seen again for a period of 7 months and beginning October 31, 1938, 25 mg. of testosterone propionate was given 6 times a week. At the end of the second week he had frequent erections and satisfactory intercourse, however, without ejaculations. No further injections were given for a period of 4 weeks. They were again resumed 3 months later (February 1939) and continued 3 times a week until July 3.

It is of interest that hair returned on his scalp, it came in black and then turned grey within a short time (4 weeks) (fig. 2). Facial hair also increased in amount so that he shaved 3 times a week. The photograph shows the appearance of the patient after 420 mg. of testosterone propionate (fig. 3).

In December 1939, he again received therapy after a lapse of 6 months. In the interval he stated that he retained his libido and potency for but one month after discontinuance of therapy.

On March 9, 1940, 450 mg. of testosterone propionate (150 mg. to each pellet) was implanted by the Deanesly and Parkes method in the left upper rectus region.

COMMENT

Why should one castrate lose all libido and react psychically in an abnormal way and another castrate retain potency and react psychically as a normal male?

As Feinier and Rothman (1) state, "After puberty and adolescence with the development of the male sexual characteristics, castration need not result in the loss of libido and potency, nor are prominent mental or physical changes a necessary aftermath. Retention of sexual power probably depends to a considerable extent on the subject's preoperative mental characteristics, type of personality, pattern and tendency either to resign himself passively to his sexual defect or else combatively to compensate for it."

In my opinion there are certain organic changes not alone in the pituitary, as has been amply shown, following castration, but what would seem equally as important, changes in the hypothalamic centers. An assumption of hypothalamic involvement as well as of the pituitary would give a much more rational explanation of character and psychic changes which are met with following castration.

Grinker (2) said, "Related directly to the hypophysis and indirectly to other glands of internal secretion, the hypothalamus

1) regulates endocrine functions and their interrelations,

2) regulates and integrates conservating autonomic functions,

[1] My thanks are due to Dr. Max Gilbert of the Schering Corporation for the generous supply of this material.

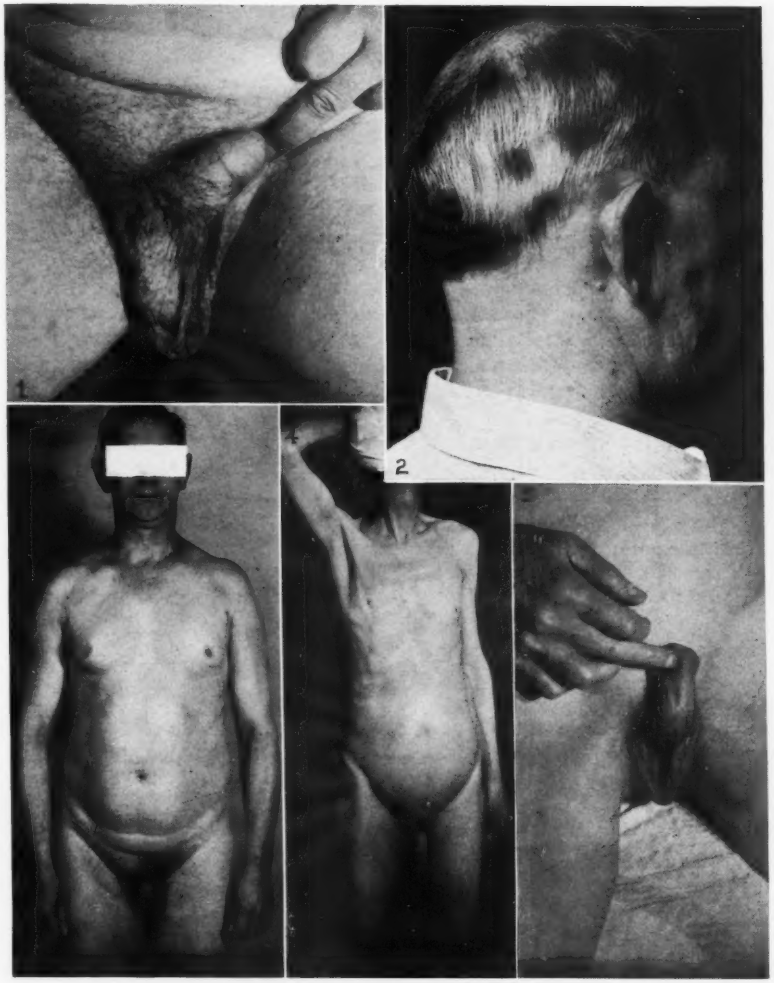

Fig. 1. Fibrous testicular remnant on right side. *Case 1.* Fig. 2. Hair growth on scalp. *Case 1.* Fig. 3. Appearance of patient. *Case 1.* Fig. 4. General appearance of patient. *Case 2.* Fig. 5. Showing absence of testicles. *Case 2.*

3) is concerned with teleologically defensive and protective reactions which we term emotional expressions and
4) influences the activity of the cerebral cortex in regulation of degree of wakefulness and excitation."

The close connection between the pituitary and hypothalamus has been amply proved. Fisher, Ingram and Ranson (3) have shown that a lesion in the median eminence of the hypothalamus results in degeneration of the infundibulum of the posterior pituitary.

Impulses from the emotional center in the hypothalamus may therefore affect the pituitary function and vice versa. As is well known, women at or before menstruation are often mentally upset and the involutional melancholia of the menopause may have its explanation in the hypothalamic changes following the artificial or natural menopause. Suggestive also is the increased hyperglycemia in diabetics following excitement or psychic upsets leading one to invoke the hypothalamico-pituitary system as its cause.

"Psychogenic fever," increase of blood pressure and many more clinical problems may well find an explanation in disturbance of this system.

Differences in individual psychic make-up are probably due to constitutional factors. Kretschmer (4) in his book "Körperbau und Charakter" has emphasized the psychoses as they affect people of certain body build. The sthenic built individual is predisposed to manic-depressive psychosis and the asthenic to paranoia. Shakespeare had these constitutional make-ups with their respective mental characteristics well labeled when he said, "Let me have men about me that are fat, sleek-headed men, and such as sleep o' nights. Yon Cassius hath a lean and hungry look; he thinks too much; such men are dangerous."

The former with the tendency to harm only himself, not infrequently makes his exitus by committing suicide; whereas his lean, asthenic brother, with great introspection, with schemes and conspiracy against his fellow-man, has his exitus by natural means and not infrequently commits murder; so suicide and homicide are their respective tendencies.

The cartoonist, based on empiricism, has for years always caricatured the reformer as the asthenic type with the lean, slender body, long bony fingers, and the long nose.

It would seem that the center for libido is activated by an interplay, or balance, between the testes, or ovaries and the hypothalamico-pituitary system, modified undoubtedly by the cortical centers.

A chemical interplay between the gonads and these systems would seem the logical explanation for the signs and symptoms resulting from a disturbance in any of the component members. Castration, or primary hypogonadism, naturally results in definite changes in the pituitary and no doubt the hypothalamic system is equally affected. In those patients in whom potency is retained, as stated by Feinier and Rothman, and others mentioned in their report, the pituitary-hypothalamic system remains in a state which indicates activity of such nature that libido is retained and no change in the psychic make-up results.

Those, on the other hand, who lose their libido have a change in masculine personality, develop feminine characteristics and mannerisms, and have a pituitary-hypothalamic system change which indicates an underactivity.

It has been my observation that true congenital hypopituitarism in the male results in a feminine build, feminine characteristics and a psyche closely simulating that of the female. Naturally the relationship this condition has to homosexuality is a close one, if not actually etiological. In these individuals the pituitary-hypothalamic system is primarily at fault and the testicle is secondarily involved.

Certain clinical facts show that the pituitary is responsible for the body build. For instance, there is every reason to believe that it is the pituitary influence on the cartilage of the larynx that has to do with its development. A lack of pituitary secretion during the developmental period of life, prepubertal and pubertal, results in a small, underdeveloped larynx with the consequent high-pitched voice rightly associated with feminism. Further proof of the pituitary's influence on cartilage, and

in this instance the larynx, is shown in the hyperpituitarism of acromegaly. In this disease the female voice changes to the masculine with a bass-pitched tone.

Of course the pituitary influence on cartilage and skeletal development is well known.

Hyperpituitarism in the female results in masculinity, with the aforementioned deep-pitched voice, hirsutism, amenorrhea, atrophy of the breasts; and not infrequently is associated with homosexuality. The inference is that the pituitary changes produce a concomitant change in the neighboring hypothalamus.

That organic changes are responsible for the psychic changes is shown by the fact that the patient herein reported did not lose his libido and potency for two years after the partial removal of the second testicle. Coincident with the loss of libido and potency, certain organic changes appeared, loss of scalp hair, beard, axillary and pubic hair, and arthritis. Presumably, the small testicular remnant had at this time undergone complete atrophy followed by both psychic and somatic changes. The arthritis is seemingly similar to the menopausal form of arthritis in the female.

Case 2. C. G., aged 58, single, American, "artist," admitted May 23, 1930 with the complaint of habitual headaches and impotency of 30 years' duration.

He stated that 30 years previous to admission both testicles were removed following an operation to produce sterilization. His story was a bizarre one. After getting his confidence during the stay in the hospital, he gave his story as follows: He had been a shoe clerk previous to being an "artist." In putting on the shoes of female clients he always had an orgasm. This was so distressing that he requested a surgeon to sterilize him. This the surgeon did and both the wounds and testicles became infected, necessitating bilateral orchidectomy. He stated that he sued the surgeon but "he was too high up in Masonry" and the patient lost the suit.

Within two months after castration he developed severe headaches. Libido and potency were also lost about 3 months after castration. He had palpitation of the heart and spells of fainting. He had a ravenous appetite but did not gain weight. He gave up his occupation as a shoe clerk and became an artist, presumably a portrait painter although he never displayed any of his work. He worked at this occupation until 1918 and apparently, because of failure to maintain a living, he became a public charge, entering and reentering the Eloise County Farm and other similar institutions on several occasions.

He stated that his mother was living (age not given) at the "Old Soldiers' Home." His father died of gastric carcinoma. One sister died of tuberculosis, and one brother died of heart disease. The patient had had typhoid fever and pneumonia in childhood.

Physical examination. The patient was a slender, anemic looking male, with a smooth, feminine type of skin. His mannerisms were distinctly feminine, a "perfect lady" would characterize him.

There was absence of hair on his head, eyebrows, face, axilla and body, and but a sparse amount on the pubis (fig. 4, 5). The gums were edentulous.

The thyroid gland was symmetrically enlarged. The testicles were absent. No operative scars were present in the inguinal region such as are seen in sterilization operations. The penis was normal in size (see photograph). The prostate was very small and atrophic.

X-rays of the skull gave normal findings and the sella turcica was normal in size.

The blood count showed hemoglobin 70%, erythrocytes 3,060,000; leucocytes 9000; polymorphonuclear neutrophiles 81%, emphocytes 18%, eosinophils 1%. The urine, other than an occasional pus cell, was negative.

The dextrose tolerance test was as follows.

Fasting	117 mg.
¾ hr.	143 mg.
1½ hr.	117 mg.
2½ hr.	105 mg.

No glycosuria was present during the test.

The blood Wassermann test result was negative. The blood nitrogen was 33 mg.

The B.M.R. was +13%, and 3 days later it was +25%. His temperature ranged from 97° to 100°. The blood pressure was systolic 114, diastolic 66.

He was discharged 6 days after admission, as he was too unruly and uncooperative. The interne's progress note read, "Patient very uncooperative, complains continually because the doctors seeing him are not responsible enough. Eats immense quantities but states that he is not getting enough to eat."

COMMENT

It is quite apparent that this patient was a psychopath. It would seem reasonable to assume that the surgeon castrated him because of some pathological condition, possibly testicular tuberculosis since a sister died of tuberculosis. His statements concerning potency and libido are not reliable, although the headache and fainting attacks were seemingly genuine. The dextrose tolerance test showed an increased tolerance, indicating a hypofunction of the pituitary.

The basal metabolic rates do not seem to be true readings, because of poor cooperation. The thyroid gland was enlarged but he showed no clinical evidence of hyperthyroidism.

No treatment was given since no satisfactory medication had been developed at that time (1930).

SUMMARY

Two patients who were castrated are reported. The first had a small remnant of testicle remaining and this seemed adequate enough to supply male sex hormone for 2 years after castration. Coincident with the loss of libido and potency, there developed total alopecia, loss of facial, axillary, truncal and pubic hair.

Thirteen years after castration treatment with testosterone propionate resulted in a return of libido, potency and a psychic change. Small doses (10 mg.) of the hormone were of no benefit, but the larger doses (25 mg.) were very effective. The first patient had implantation of 450 mg. testosterone propionate and within 10 days libido, potency and frequent erections had occurred.

A second castrate, who was a psychopath, lost his libido, potency and apparently changed psychically soon after castration. Castration had been performed 30 years previously and the individual had a psychic pattern resembling the female.

The suggestion is made that there is a chemical balance, or interplay, between the gonads and the pituitary-hypothalamic system, and it is the change in the latter system which determines the extent of libido, potency and psychic pattern.

That testosterone propionate is a potent remedy and supplies the male sex hormone seems well established. Ketcham (5) and Turner (6) report good results from the use of testosterone propionate. Howard and Vest (7) reported observations extending over a period of 2 years on the use of testosterone propionate by the parenteral route in 20 hypogonad adults with very satisfactory results. More recently they have implanted the pellets of the synthetic male hormone with satisfactory results.

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SEX HORMONE CHANGES ASSOCIATED WITH LIVER DISEASE[1]

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SINCE June, 1938, we have studied 14 males hospitalized, primarily, for chronic liver disease. Gynecomastia in 8 and testicular atrophy in all of the cases prompted us to investigate the sex hormone metabolism in addition to the usual clinical studies in these men. We are reporting here the results of urinary bioassays in these, as well as in a significant number of control subjects, including one case of subacute liver cirrhosis. Detailed clinical and postmortem data of patients who died of liver failure will be reported elsewhere.

Our preliminary report (1) last year suggested that estrogen metabolism was impaired in advanced cirrhosis of the liver. The present data supplement and confirm our previous findings and also provide a theoretical basis for a hormonal etiology of gynecomastia in chronic liver disease.

Since Zondek (2) enunciated the theory that the liver inactivates sex hormones, ample experimental proof of such a mechanism has been brought forward by a number of workers (3–7). Our data also tend to show that the human liver manifests this function of hormonal inactivation.

Inasmuch as most investigators (8, 9, 10) find little or no biologically free androgens or estrogens in urine bioassays, the recovery of these sex sterols in free rather than in combined form may be considered as evidence that the liver has failed to inactivate these compounds. To put this theory to test urine specimens from the fourteen "liver" cases were subjected to bioassay to determine the free and combined fractions of the androgens and estrogens.

Since the majority of these patients suffered from advanced liver cirrhosis associated with ascites and other evidence of liver insufficiency the recovery of considerable free sex hormones in the urine is indicative perhaps, of a more specific functional failure.

METHODS

Six-day pooled and preserved urine specimens were promptly assayed biologically for estrogens and androgens. To determine the combined or total and the free fractions the specimens were separated into 4 parts, 2 of which were assayed after the usual extraction process which included 15-minute acid hydrolysis for the total androgen fraction and 2-hour hydrolysis for the total estrogens. The other 2 fractions were similarly assayed but without the preliminary hydrolysis of the urine. The omission of hydrolysis makes available what is considered the free fractions of estrogens and androgens. This method was applied in 9 of the liver and 12 of the control subjects. No such "fractionation" was carried out in our first 5 liver cases (Series I).

[1] Read at the twenty-fourth annual meeting of the Association for the Study of Internal Secretions, New York City, June 1940.

TABLE 1. NORMAL SEX HORMONE VALUES IN MALE URINE, 24 HOURS

Patient	Age	Androgens		Estrogens	
		Free	Total	Free	Total
		I.U.	I.U.	γ	γ
M.G.	31	0	26	0	9.0
R.F.	26	0	48	0	4.5
H.H.	44	0	30	0	4.5
M.L.	23	0	34	0	3.0
L.K.[1]	57	0	6	3	3.0
C.C.[1]	50	0	4	0	0
B.M.[1]	55	0	20	0	0
J.M.[1]	58	0	10	0	4.5
D.K.[2]	56	0	12	0	0
Range normal males (31)	22–64	Not tested	20–47	Not tested	1.5–10.5

Bioassays in winter months
[1] Hospitalized for fracture
[2] Hospitalized for peptic ulcer

Capons and spayed rats were used as previously described (1) following, with slight modifications, the methods of Koch (8) for androgens and Gustavson (11) for estrogens. Incidentally it was found that extraction of the concentrated urine by refluxing twice under chloroform gave maximum hormone yields.

RESULTS

The data depicted in the tables show marked deviations in the sex hormone values found in the cases with cirrhosis of the liver. The two most striking changes are the low androgen values (in keeping with the testicular atrophy) and the presence of practically all of the estrogens in free form in the majority of the "liver" cases where "fractionation" was carried out. Noteworthy, too, is the appearance of considerable free androgens and estrogens in the patient with acute cirrhosis. The control subjects show a conspicuous absence of free hormone in the urine with the ex-

TABLE 2. SEX HORMONE VALUES IN GYNECOMASTICS WITH ADRENO-GENITAL SYNDROME

Patient	Age	Total androgens	Total estrogens	Comment
		I.U.	γ	
H.G.	48	90	16	No adrenal tumor
E.R.	39	96	450	Adreno-cortical carcinoma

TABLE 3. SEX HORMONE VALUES IN GYNECOMASTICS (WITHOUT KNOWN LIVER DISEASE)

Patient	Age	Androgens		Estrogens	
		Free	Total	Free	Total
		I.U.	I.U.	γ	γ
H.H.	60	0	0	2.5	2.5
D.B.	19		20		4.0
D.B. (Repeat)	20	0	24	0	4.5
J.D.	15		6		9.0

TABLE 4. SEX HORMONE VALUES IN CHRONIC CIRRHOSIS OF LIVER, SERIES I

Patient	Age	Total androgens	Total estrogens	Atrophy, testes	Comment
		I.U.	γ		
J.M.	12	32	9.0	++	Died with gynecomastia and adreno-cortical hyperplasia at p.m.
F.R.	52	60	36.0	++	Died (same findings as above)
J.A.M.	47	28	4.5	+	Gynecomastia
R.C.	54	0	12.0	+	Gynecomastia
T.P.	32	26	10.5	+	Questionable breast enlargement.

ception of 2 elderly men, one a gynecomastic with 1.5γ and the other (*L.K.*) with 3γ of free estrogens in their urine. With two exceptions (*D.A.* and *A.S.*) the gynecomastics excreted relatively large quantities of free estrogens.

DISCUSSION

Apparently chronic or extensive liver disease results in abnormal excretion of sex hormones. Particularly interesting is the recovery of the total estrogens in free form in 8 instances of hepatic cirrhosis. In other words inactivation of the estrogen appears not to have taken place.

The liver damage in the acute case (M.C.) must have been extensive enough to manifest partial failure to inactivate both androgens and estrogens. Thus, this bioassay approach might serve as a sensitive measuring stick of hepatic function in questions of liver insufficiency. We are investigating this possibility.

Gynecomastia and testicular atrophy are striking concomitants of liver cirrhosis in the majority of cases. This curious syndrome has been described only in foreign literature (12). Corda (13) implied that the gynecomastia in liver cirrhosis is of endocrine origin. Our findings add much weight to such an etiology. It seems logical to relate the mammary growth to the factor of free estrogens seen in 5 or 6 out of the 9 instances where free fractions were determined. That there may be other contribut-

TABLE 5. SEX HORMONE VALUES IN CHRONIC CIRRHOSIS OF LIVER, SERIES II

Patient	Age	Androgens		Estrogens		Gynecomastia	Atrophy, testes
		Free	Total	Free	Total		
		I.U.	I.U.	γ	γ		
C.F.	55	0	6	10.5	10.5	Present	+
E.L.	48	0	0	15.0	15.0	Present	++
J.C.	55	0	0	6.0	6.0	Present	++
F.K.	59	0	0	15.0	15.0	Present	++
H.H.	35	0	4	10.5	12.0	Present	+
D.A.	59	0	8	0	3.0	Absent	+
A.S.	48	0	8	0	1.2	Absent	++
B.P.	39	(1)0	4	10.5	10.5	Absent	+
Repeat		(2)0	0	15.0	15.0		
				Subacute Cirrhosis			
M.C.	26	20	32	4.5	12.0	Absent	—

ing factors to this sex anomaly is a possibility. However, the experimental evidence favors estrogens as playing the dominant rôle. It has been repeatedly demonstrated that adequate estrinization will induce mammary growth in both sexes. Certainly, the gynecomastia in other feminizing syndromes like the adreno-genital syndrome must be considered a result of the abnormally high concentrations of estrogens. This is illustrated by 2 of our control cases who manifested a consistent picture of gynecomastia in the presence of high levels of estrogens. This relationship has been noted previously (14). But of greater significance is the fact that the estrogens in our "liver" cases are in free physiologic state and in this active biologic form can be more potent in relatively lower concentrations.

SUMMARY

This study may be regarded as serving a dual purpose: first, to add support to the theory that the liver inactivates the sex sterols and secondly, to indicate a possible pathogenesis for the gynecomastia seen in 8 out of 14 men with cirrhosis of the liver.

Bioassays yielded no combined but high free estrogen values in all the advanced cases of liver cirrhosis.

Low or negative androgen values were found and seemed to harmonize well with the testicular atrophy noted in all the "liver" cases.

The data indicate a failure of the cirrhotic liver to inactivate estrogens and it is suggested that the free estrogens are etiologically related to the production of gynecomastia in advanced cirrhosis of the liver.

The bioassays were carried out in the hormone laboratory of Dr. S. J. Glass, and the authors are grateful to Mr. Levi and H. Hess for technical assistance.

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THE EFFECT OF PREGNANT MARE SERUM ON SPERMATOGENESIS IN MAN

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THE PATIENT A.R., age 32, was referred by a local urologist because of sterility. The wife had been examined by a gynecologist and appeared to be normal in every way. The couple had been married 6 years and had not used contraceptive measures for 5 years. Marital relations were normal except for a tendency to premature ejaculations at times on the part of the patient.

The past history included chicken pox, whooping cough and measles. At 10 he had mumps bilaterally but stated that there was no orchitis at that time. No history of venereal infection could be elicited.

At the time of the initial examination he felt in good health except that he tired easily and had difficulty in getting to sleep.

On physical examination he was found to be a tall slender but well nourished young man and there were no signs or symptoms of systemic disease. The urological examination results were entirely negative as were those of the blood and urine examinations. The basal metabolic rate was slightly elevated. A semen specimen measured 4 cc. and showed rare sluggishly motile spermatozoa at 1 hour and no motility at 2 hours. The differential count was as follows: normal 52%, pyriform 16%, microform 14%, macroform 4%, abnormal acrosomes 6% and other abnormalities 8%. The count showed 32,500 spermatozoa of all types per cu. mm.

Treatment was instituted consisting of injections of pregnant mare serum[1] 3 times weekly. For the first 2 weeks the amount given was 5 Cartland units and then the dose was increased to 10 U. These injections were continued with rest periods of one month for 2 years as shown in table 1. In addition to this treatment the patient was also given ½ gr. daily of thyroid (Armour), Vitamin ABD & G concentrate and wheat germ oil, and this medication was taken continuously throughout the entire period.

From the institution of treatment gradual progress was shown by the decrease in abnormal types and increase in number of spermatozoa. At the same time the vigor, activity and viability of the spermatozoa showed marked improvement, so that whereas the first samples contained but a small percentage of active spermatozoa which showed a sluggish motility lasting for only one hour the later specimens showed a high percentage of vigorously motile spermatozoa and this acticity lasted for about 72 hours when allowed to stand at room temperature.

An examination of the table will also show that the spermatozoa count tended to fall during the periods in which the injections were discontinued. After one year of treatment the specimens were considered to be normal as the counts were consistently over 100,000 with less than 20% of abnormal forms and the motility was excellent. At this time the patient stated that he felt better than at any time during his married life and had ceased to have any premature ejaculations. Artificial insemi-

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[1] The preparation used was gonadogen supplied through the courtesy of Dr. E. Gifford Upjohn of The Upjohn Company.

TABLE 1. EFFECT OF PREGNANT MARE SERUM ON SPERMATOGENESIS

Date	Count per cu. mm.	Abnormal types of Sperm.	Motility		Medication
			Activity	Duration	
1938		*per cent*			
Mar. 9	32,500	48	Very sluggish	1 hr.	Gonadogen 5 U. 3×week Thyroid gr. ss daily Vitamin concentrate ABDG. Capsules Wheat germ oil
Apr. 9	36,000	40	Very sluggish	1 hr.	Gonadogen increased 10 U. 3×week
Apr. 30	42,500	38	Very sluggish	1½ hr.	Gonadogen increased 10 U. 3×week
May 14	57,000	33	Slightly better	1½ hr.	Gonadogen increased 10 U. 3×week
May 28	56,000	27	Definitely better	2½ hr.	Gonadogen increased 10 UO 3×week
June 11	60,400	26	Less than last exam.	2 hr.	Gonadogen increased 10 U. 3×week
June 25	50,600	32	Markedly improved	2½ hr.	Gonadogen increased 10 U. 3×week
July 9	48,800	30	Best since start of treatment	2½ hr.	Gonadogen discontinued. Other medication retained
Sept. 10	45,800	29	Excellent	2–3 hr.	Gonadogen 10 U. 3×week
Oct. 23	64,600	23	Vigorous	3–4 hr.	Gonadogen 10 U. 3×week
Nov. 12	57,800	21	Vigorous	3–4 hr.	
Nov. 29	70,000	28	Vigorous	4–5 hr.	
Dec. 13	Specimen lost				Injections discontinued. Other medication retained
1939					
Jan. 21	58,600	23	Vigorous	4–5 hr.	Gonadogen 10 U. 3×week
Feb. 4	102,200	20	Vigorous	5–6 hr.	Gonadogen 10 U. 3×week
Mar. 4	87,200	19	Excellent	5–6 hr.	Gonadogen 10 U. 3×week
Apr. 17	109,800	18	Excellent. Some motility after 24 hr.		Gonadogen 10 U. 3×week
May 15	128,800	17	Very vigorous. Some motility after 48 hr.		Rest period
June 17	89,400	19	Very vigorous		Gonadogen 10 U. 3×week
July 22					No treatment for one week
July 29	117,600	21	Very good but numerous non-motile sperm.		Injections renewed. Gonadogen 10 U. 3×weekly
Sept. 9	85,000	18	Excellent		Injections renewed. Gonadogen 10 U. 3×weekly
Oct. 7	Specimen spoiled.				Injections discontinued
Nov. 4	116,000	15	Very vigorous	69 hr.	Gonadogen 10 U. 3×weekly
Dec. 9	111,200	18	Very vigorous	72 hr.	Gonadogen 10 U. 3×weekly
1940					
Jan. 13	130,400		Excellent		Gonadogen 10 U. 3×weekly
Feb. 3	133,800	16	Very vigorous	>48 hr.	Injections discontinued
Mar. 7	78,200	15	Very vigorous	>48 hr.	

nation was tried in July 1939 on the 12th day of the cycle and again in September on the 14th day of the cycle. At this time a Rubin test for tubal patency was asked of the consulting gynecologist but on his advice the couple decided to wait another 6 months before the test was performed.

The test was performed recently and showed conclusively that both tubes were blocked. Insufflation with air to 220 mm. Hg was maintained, and salpinography with iodized oil showed the tubes open only for about 1 cm. from the uterus. Because of this all further treatment of the husband was discontinued.

The case is presented as it adds one more bit of evidence as to the beneficial effect on spermatogenesis produced by extracts of pregnant mare serum, and also because of the fairly rare occurrence in which defects in both partners were found either of which alone would have been sufficient explanation of the sterility. It also shows again the necessity for ruling out any abnormalities in the second partner when treatment has overcome the deficiencies found in the first partner and the sterility still persists.

THE ENDOCRINE DYSCRASIA OF ACNE VULGARIS IN WOMEN[1]

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IN 1936, one of us published a report on the use of pregnancy urine extract (1) in the treatment of acne. In that report it was stated that the available evidence indicated an endocrine functional imbalance as the probable cause of the condition, and the necessity for further study was stressed. Such study has been retarded by lack of effective methods of hormone assay, but during the past year we have carried out quantitative measurements of estrogen and androgen excretion in 8 normal women, and in a similar number who had acne. Publication of the results in so small a series is justified by proof of the validity of the differences between the values under discussion, which proof was obtained when the data were subjected to statistical analysis (15).

During the past 4 years several papers on the etiology of acne have appeared (2, 3, 4). The subject has been recently reviewed so completely by Stokes and Sternberg (2) that there is little value in analyzing the literature further in this report. We have found no recent publications which appear to vitiate their conclusions. Wile, Snow and Bradbury (3) and Cornbleet and Barnes (4) have examined the urinary excretion of sex hormones by acne patients, and have compared them with the excretion of normal individuals.

These workers investigated both male and female cases, while this report is confined to the study of females. Preliminary work on males indicates a similar dyscrasia, but the quantitation of the estrogenic fraction is more difficult than in female urine extracts, and the variability introduced is so large that significant results would not be obtained from a small series of assays.

In the series to be presented, urine studies have been made on 8 normal individuals and 8 patients with acne. The patients were referred to our Endocrine Clinic from the other clinics of the Medical Center and so may not represent a true aliquot of acne vulgaris. Each case is associated with severe dysmenorrhea.

METHODS

Because of the amount of labor requisite to the analysis of a urine specimen we chose to limit each patient to one 48-hour specimen taken between the 16th and 20th day of the menstrual cycle. This time period was chosen because it presumably embraces the period of high hormone excretion (5). The criteria of normality in our control specimens is a history of regular cycles, 5-day flow period, and a complete lack of dysmenorrhea. These criteria have of necessity greatly limited the number of normal subjects.

(*a*) A 48-hour urine specimen was collected between the 16th and 20th day of the menstrual cycle.

[1] This work was supported by the fund donated by the Bingham Foundation.

(*b*) Fifteen-hundred cc. was acidified to a pH of 1, brought to a boil, cooled slightly (10–15°C.), and extracted with toluene for 2 hours on our continuous extractor. (This extractor is essentially that recently described by Talbot (9) except that a paddle is introduced from the top. The advantage over the Talbot type is that it is less subject to wear.) The use of toluene provided a simultaneous hydrolysis and extraction, since the temperature at which the toluene condenses is high enough to maintain the urine at 80°C. during the extraction.

(*c*) At the end of 2 hours the urine-toluene and emulsion is collected and allowed to separate into 2 layers (toluene with emulsion and urine). The urine is discarded.

(*d*) The toluene and emulsion is then boiled under a modified reflux condenser which permits separation of the water from the condensate and the return of the toluene to the flask. This was done until the toluene was dry and the water-soluble materials were out of solution.

(*e*) The toluene was then filtered into a separatory funnel (1 liter pear-shaped).

(*f*) It was extracted 3 times with 0.1 N NaOH. This was termed the estriol fraction, but it contained some estrone and some estradiol.

(*g*) The estrone and estradiol were removed by backwashing once the 0.1 N NaOH with its own volume of peroxide-free ether. Toluene was added to the ether after separation and the ether evaporated off on the steam bath.

(*h*) The toluene remaining and holding the organic material in the backwash in solution was then added to the original toluene.

(*i*) The toluene was extracted with 1 N NaOH 3 times. This was termed the estrone fraction. It also contained estradiol.

(*j*) The 0.1 N and the 1 N NaOH solutions were acidified to pH 1 and stored under toluene until extracted for 2 hours in the continuous extractor.

(*k*) The original toluene was now called the male fraction. All the toluene fractions were evaporated to dryness in vacuo.

(*l*) Each fraction was then taken up in ether (200 cc.).

(i) Estrone and estriol fractions were shaken with successive washings of a saturated Na_2CO_3 solution until no dirt showed at interface, then washed with distilled H_2O to remove Na_2CO_3, evaporated and the residue taken up in 10 cc. 95% ethyl alcohol.

(ii) The male fraction was treated with 2 gm. of Norit A for exactly 4 minutes in 200 cc., ether filtered, Norit washed with 50 cc. additional ether, ether evaporated, taken up in 20 cc. 60% alcohol and refiltered into storage tubes. The final solutions were always colorless.

Androgen assay. (*a*) One, 2 and 3 cc. of colorless sample were placed into 3 absorption tubes (Cenco photolometer, shell vial absorption cells).

(*b*) The 1 and 2 cc. samples were made up to 3 cc. with 60% alcohol.

(*c*) One cc. of a saturated solution of metadinitrobenzene in 60% alcohol and 1 cc. of 15% KOH solution was added.

(*d*) The vials were kept 5 hours at 25° and read between the fifth and sixth hours until consecutive values approximated one another.

Androgen standard. Made by pooling androgen fractions of 20 patients. Then aliquots were taken as 0.1, 0.2 and 0.3 cc. and a calibration curve obtained based on arbitrary units (0.1 cc. = 1 unit). The curve was found to be different for male and female patients.

For determining the content of the estrogen fractions we used a modification of the Astwood technic involving intraperitoneal injection of 0.2 cc. of the unknown in oil. In general we find 4 animals at one dose adequate for clinical work. (The maximum error possible with a frequency of once in 100 trials = 40%) (6). The basis for the above extraction and evaluation technics is to be found in the papers of Smith, Smith and Schiller, and Nathanson (7, 8).

The data obtained by the use of this technic are given in table 1. Inspection of the table shows that the average value of the ratio androgen units/estrone equivalent in gamma is higher for the acne patients than for the normal individuals. The calculation of the reality of this difference shows that the probability that the two groups (acne patients and normal controls) belong to the same group is less than 1 in 100. It is also possible to show a real difference between the average estrone equivalents in the two

groups. The variability of the values of the control subjects is high. This high variability is expected since a peak in estrogen excretion has been demonstrated (5). It is too much to expect that each of the control subjects should be at the same point in their changing estrone excretion.

By using the logarithm of the estrone value to reduce the inherent variability it is possible to show that a real difference also exists between these two groups.

If, as can be seen by inspection, the androgen values are the same for the two groups—then, if the estrone values are different the ratios must also be different.

TABLE 1

Patient	Androgen value	Estrone equivalent, γ	R = Androgen / γ Estrone equivalent
	Normals		
M.L.	170	58	2.9
	198	65	3.0
E.E.	152	65	2.3
M.	100	60	1.7
A.	95	50	1.9
B.	126	50	2.5
K.	260	170	1.5
P.	328	85	3.9
Averages	178	75	2.46
	Patients with acne		
F.	72	12	6.0
A.G.	140	20	7.0
C.	107	20	5.3
K.	144	32	4.5
W.	220	32	6.9
B.	241	36	6.7
Wel.	226	20	11.3
Br.	120	21	5.7
Averages	158	24	6.67
Dif. between av's.	20	51	A/E 4.21

t = 5.26 for R; t = 6.00 for log. estrone equivalents.
(For 14 degrees of freedom at value for probability of one in a hundred = 2.977.)
Thus there is a probability of less than 1 in 100 that samples were drawn from same population for both R and estrone values (Statistical Method, Snedecor, G. W. Collegiate Press Inc., Ames, Iowa, 1938).

We have chosen, however, to place more clinical weight on the altered ratio than on the disparity between the estrone values. Two observations in the literature induce us to take this view.

The first is the fact that women with adrenal tumors of the masculinizing type, frequently show acne (10). In these patients the lack of balance between the two hormone types is obvious. Several workers have shown that the administration of testosterone to women will induce the appearance of acne (11, 12).

The great difference in the methods employed and the resulting difference in the yield of the extraction procedures renders it impossible to compare the results obtained by Wile, Snow and Bradbury with our own observations, nor can we be certain that the values obtained by our technic are the true absolute values of the hormones excreted in the urine. We have used the recovery data of previous workers to justify the technics used. But to date it has been impossible to add to urine hormone in the form in which it is excreted, and demonstrate the percentage recovery under such

ideal conditions. Furthermore the possibility always exists that the concentration of interfering materials in any particular urine may cause a significant deviation in the percentage of material extracted. This latter point, however, is an obstacle encountered in practically all laboratory procedures.

In view of the factors outlined above, the point that we obtained from our acne patients, an average of 24γ estrone equivalents in comparison to 12 as found by Wile et al. (3) cannot be taken to indicate that the group studied by them differed materially from ours.

Since the two groups were in all probability not different in so far as they were aliquots of the acne population it is reasonable to compare the findings obtained by the studies on the two groups.

Our data agrees with theirs in that the urinary estrone equivalent is lower in the acne group than in the normal. The findings of a higher ratio of androgen/estrone for the acne patients is also in agreement.

But we disagree with the findings of Cornbleet and Barnes (4), showing a decreased androgen output. Wile et al. found a heightened androgen excretion (3). The discrepancies can be resolved, however, by subjecting all the data to statistical analysis. Statistical treatment demonstrates, on the basis of the data presented, that in all the series it is invalid to conclude that either an increased or decreased androgen excretion occurs. A difference may really exist, but the evidence is inadequate to establish such a difference.

SUMMARY

A significant decrease from the normal in the urinary estrogen excretion by women with acne was demonstrated. No significant change in excretion of urinary androgenic material was observed. A significant increase in the ratio of androgenic material to estrogenic material measured as estrone equivalents was demonstrated.

Several observations were quoted to indicate that more clinical weight should be given to the aberrant ratio than to the decreased estrone excretion as the major etiological factor. In view of these findings the restoration of a normal ratio would appear to be an important consideration in the treatment of acne.

From the clinical point of view, we believe that it is significant that the findings in our series agree with the biological evidence concerning acne. This evidence comprises the characteristic limitation of acne to the pubertal period; the frequent association of the eruption with abnormal menstruation; the reports in the literature of the appearance of acne in females being treated with testosterone followed by its disappearance when the treatment was omitted. All this evidence is consistent with the thesis that in the female, a disturbance of the androgen estrogen ratio is a significant etiological factor in acne.

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BLOOD SUGAR STUDIES IN A CASE OF ADIPOSO-GENITAL DYSTROPHY SHOWING CHRONIC HYPOGLYCEMIA

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IN THIS COMMUNICATION we report the results of blood sugar studies made on a case of adiposo-genital dystrophy that showed a chronic hypoglycemia. The patient was under observation for a total of 16 months in the New York City Hospital, Welfare Island, 2 months of which was in the general medical ward and the rest, 14 months, in the metabolic section. Our main object in the investigation was to find some therapy that might raise the prevailing subnormal blood sugar values to a normal level. Reasonable opportunity was given to several diets and to those commercial endocrine products stated to be diabetogenic in nature.

CASE REPORT

C. R., a male, 27 years old, entered the hospital, May 29, 1938, for obesity and periumbilical pains. His weight at that time was 369 lb. and his height was 67½ in. He met all the requirements of Fröhlich's syndrome; excessive obesity, pubic distribution of fat, a high sugar tolerance with loss of libido and potentia. His B.M.R. was normal and his blood pressure was somewhat raised at that time. (165/90). He was kept in a general medical ward and put on a diet of 1000 calories with C 84, P 110 and F 25. On June 13 a sugar tolerance test with 100 gm. of glucose was 85, 114, 98, 90, 66.

He was transferred to the metabolic service on August 5. He then weighed 155.4 kg. (343 lb.) His before breakfast sugar on that date was 63 while one hour after the meal it was 86. His breakfast consisted approximately of C 30, P 40 and F 10. He complained very much of waking at about 2 A.M. with hunger and not being able to sleep the rest of the night because of weakness, nervousness and vague abdominal pains. This was usually relieved by breakfast. He had very few such feelings during the day. Rather than have him cheat we raised the total daily calories to 1600 with the largest meal at night. At the same time we lowered the carbohydrates and raised the proteins and the fats to make a diet of C 33, P 39, F 28.

On August 6th he was given insulin, 5 U, before breakfast and this was raised 5 U a day. He shocked mildly in the forenoon after he reached 20 U. His blood sugar on August 13, two days after the insulin was stopped was a.c. 74 and one hour p.c. 61.

On August 15 he was given 1½ grains of thyroid extract a day and on August 23rd the blood sugar was a.c. 87 and p.c. 78. The thyroid was then raised to 2 grains a day and on the 28th the blood sugar was 93 a.c. and p.c. By September 6th the thyroid was raised to 4½ grains and on the 7th the blood sugar was 93 a.c. and 98 p.c. On the 8th the thyroid dose was 8 grains a day. That day ½ ampule of obstetrical pituitrin was given before each meal. There were no changes in the blood pressure, so on the 10th the dose was increased to a full ampule. This was continued through the 19th with no noticeable effect on the blood pressure. It was stopped on the 20th because for the last few doses he was getting weak spells with intestinal cramps and a desire to move the bowels a short time after the injection. When he defecated he was relieved. His blood sugar on the 20th was a.c. 73 and p.c. 85. By this time the thyroid had been gradually increased to 27 grains a day. The patient had no complaints although his pulse averaged about 130 per minute. It was decided to leave the dosage at this point for some time unless disagreeable symptoms set in.

On October 5th one ampule of adrenalin was started before each meal. The blood pressure was taken before the adrenalin was given and was found to be 144/84. It was again taken

one hour after the injection and it was 144/70. A daily blood sugar curve was taken on October 10th. At 7 A.M. it was 62, 11 A.M. 77, 4 P.M. 83 and at 9 P.M. 91. On October 13th the blood sugar was a.c. 63 and p.c. 103. On the 14th the adrenalin was stopped and a blood sugar test made before breakfast was 60 and one hour after the meal was 69. We then started anterior pituitary by mouth at the rate of 2 grains t.i.d. October 18th the blood sugar was a.c. 95 and p.c. 81. By October 24th the pituitary dosage had reached 24 grains a day and a blood sugar on that date was a.c. 57 and p.c. 75. On October 25th the thyroid extract was reduced to 15 grains a day because it had no apparent effect on the blood sugar level and the pulse had averaged around 130 for over a month with the patient complaining about his digestion. On the 28th his B.M.R. was +7 and +4.

On November 2nd his blood sugar was a.c. 80 and p.c. 100. By the 7th the anterior pituitary had reached a dosage of 50 grains a day with 15 grains of thyroid extract. On the next day the anterior pituitary was discontinued and the blood sugar was a.c. 83 and p.c. 74. Suprarenal gland by mouth was started on the 10th with 6 grains a day. On that day a blood sugar showed a.c. 103 and p.c. 78. On the 23rd the blood sugar was 89 a.c. and 65 p. c. By December 6th the patient was receiving 66 grains of suprarenal extract by mouth each day. On December 10th the blood sugar was a.c. 95 and p.c. 100.

Starting December 15th he was given 200 R.U. (2 cc.) a day of follutein. On December 19th the blood sugar was 85 a.c. and 66 p.c. On December 31 the A.Z. test was negative.

Blood sugars measured on January 6th 1939 were a.c. 85 and p.c. 80. On January 9th a glucose tolerance test with 100 gm. of glucose gave: fasting, 85; ½ hour, 108; one hour 83; two hours, 83 and 3 hours 77. A gastro-intestinal and gall bladder series was made on the 12th and results were normal. On the 13th one ampule of antuitrin was given and this was continued until the 18th. On that day a glucose tolerance test was made with a 100 gm. of glucose. At fasting it was 62, ½ hour, 80, one hour, 60; 2 hours 66 and 3 hours, 85.

The diet was changed on January 20th to C 42, P 89 and F 146. On the 25th a fasting blood sugar was 103 and one hour after breakfast was 93. The diet was then reduced to 750 calories consisting of C 48, P 70 and F 31. Insulin 15-0-10 was then given for 6 days. On the 25th the blood cholesterol was 182 and the cholesterol esters were 100. On the 1st of February the a.c. blood sugar was 83 while one hour after it was 65.

Beginning the 2nd of February the patient was given 45 grains of ammonium chloride a day for two weeks. On the 15th the fasting blood sugar was 64 and one hour after the meal was 83. The patient left the hospital on April 17, 1939, weighing 253 lb.

He returned to the hospital August 4, 1939. He then weighed 122.6 kg. (270 lb.). Blood sugars on that date were a.c. 95 and p.c. 103. He was put on a diet of C 11, P 67 and F 206 which was 2166 calories. On August 10th his blood sugar was a.c. 108 and p.c. 114. The next day, August 11, he was put on a diet of C 2, P 22 and F 258. This amounted to 2418 calories. On August 17, the blood sugar before breakfast was 75 while one hour after it was 85. His diet then was raised to C 2, P 24 and F 322. This was the highest amount of calories, 2950, he received.

On August 21 the cholesterol was 308 while the cholesterol esters were 83. B.M.R. was −8 and −5. His weight was 122.6 kg.

Blood sugar estimations on August 24th were a.c. 118 and p.c. 114 and on the 28th the a. c. was 89 and the p.c. was 73. On September 6th his diet was changed to C 130, P 113, and F 128. His weight on September 13 was 119.9 kg. and on September 16 it was 120.

On October 7, 9, 11, and 13 he was given 1 cc. of testosterone propionate. On the last date a glucose tolerance test result was fasting, 77; ½ hour, 77; 1 hour, 75; 2 hours, 108 and 3 hours 105. On October 25 his total calories were dropped to 840 with C 44, P 76 and F 40. Benzedrine sulphate, 2½ mg. a day, was started on November 8th. His weight on that day was 114.9 kg. The benzedrine was raised on the 15th to 5 mg. a day. On the 25th his weight was 111.5 kg.

A Rose-Exton test result on November 27th was 87-87-105. Another taken on the 29th using, however, 200 gm. of glucose for each dose and carried out to the 3rd hour was 91, 91, 133, 91, 85. On the 30th a Rose-Exton test was made with the glucose given intravenously. The figures for the test result were 64, 125, 81, 91, 61.

Alimentary sugar tolerance tests were started with 150 gm. of glucose by mouth. It was found that the first spill in the urine occurred following the ingestion of 250 gm. of the glucose. On December 11 a blood sugar curve and a urinary spill curve was made after 250 gm. of glucose by mouth. After a fasting blood sugar of 91 we found a 15-minute value of 114; at 30 minutes, 116; at 1 hour, 100; at 1½ hours, 111; at 2 hours, 87; at 2½ hours, 87; at 3 hours, 52; at 3½ hours, 95; at 4 hours, 80 and at 4½ hours, 70. A urinary spill of one plus was seen at the end of an hour with a trace at the 1½, 2, and 2½-hour periods.

A starvation blood sugar curve was determined on December 15th. No breakfast was given and the blood sugar values were at 8:30 A.M., 83; 9:00, 85; 9:30, 91; 10:00, 95; 11:00, 87 and 12 noon, 82.

We gave him injections of adrenalin, 1–1000, in increasing doses until uncomfortable symptoms were noted. At 35 minims he complained of being nervous. Several days later, 1/9/40 at 10 A.M., his blood pressure was 112/74, his pulse rate, 72, and his blood sugar 73. He was thereupon given 35 minims of adrenalin. At 10:15 his blood sugar was 79. At 10:30 his blood pressure was 118/80, his pulse rate was 80 and his blood sugar was 85. At 10:45 the blood sugar was 103. Finally at 11 the blood pressure and the pulse rate came back to the first values but the blood sugar was then 118.

On 1/15/40 at 2:15 P.M. his blood pressure was 110/64, his pulse rate was 72 and his blood sugar was 78. He was then given 50 minims of adrenalin. At 2:45 the blood pressure was 120/66, the pulse rate 100 and the blood sugar was 94. At 3:15 the pressure was 124/68, the pulse was 128 and the blood sugar was 148. At 3:45 the pressure was 130/70, the pulse was 140 and the blood sugar 165. At 4:15 the pressure was 120/64, the pulse 140 and the blood sugar 156. Then at 4:45 the pressure was little changed while the pulse was 136 and the blood sugar had dropped to 149.

DISCUSSION

This patient qualified as a case of chronic hypoglycemia on two counts, (*a*) about 1/3 of his blood sugar estimations registered below 80 and (*b*) his blood sugar response to a meal or a glucose tolerance test was abnormally low and abnormally short. Out of a total of 108 blood sugar tests there were 34 that were below 80 and 19 that were below 70. In this group there were 13 fasting blood sugars below 80 and 7 below 70. The lowest fasting value was 57 while the lowest reading found at any time was 52. Then in the 25 partial tolerance tests, with fasting and one hour after breakfast estimations, we note on 13 occasions the one-hour post prandial blood sugar was the same or lower than the fasting value, with 10 of these falling into the hypoglycemic range. Furthermore, in the 4 complete tolerance tests that were obtained, we found an abnormal curve of a hypoglycemic nature. The ½-hour test showed a rise over the fasting figure of 29 mg. in one, 23 in one, 18 in another and no change in one. An average of the 4 give a rise of 19 mg. at the ½-hour mark. As the generally accepted normal sugar tolerance curve shows a rise of 40 mg. at this point it is evident this patient had an abnormally low blood sugar response to 100 gm. of glucose. Again at the end of an hour the figures were down to or below the fasting blood sugars showing the rapid return to the low values.

Except after excessive doses of adrenalin there was no recognizable change in the blood sugar following the various types of therapy employed. Apparently blood sugar values in cases of chronic hypoglycemia are stable. Because the glucose tolerance test is generally accepted to be influenced by the diet we tried various combinations of the foodstuffs. For the greater part of the time the diets were at submaintenance levels. This was because the patient came into the hospital for weight reduction. Sufficient time, however, was given to the higher calory diets for the necessary studies.

We do not feel that undernutrition played any part in the low sugar values in this case. Starvation, generally thought to be productive of reduced blood sugars,

has not acted that way in our experience. We have recently seen several cases of advanced anorexia nervosa with all fasting blood sugars above 90 mg. Harris (1) likewise found no change in starvation. Weeks and others (2) reported that the fasting blood sugars of 49 epileptics had been raised on an average of 20% after 3 weeks starvation. Yet a number of investigators have concluded that starvation is apt to predispose to a diabetic sugar tolerance curve. There was no evidence of this response in the case presented here. High carbohydrate diets usually keep the curve low, presumably by stimulating increased insulin production. Frequently repeated doses of carbohydrates may lower the blood sugar to hypoglycemic levels while prolonged fasts after high carbohydrate feedings provoke abnormally low blood sugar values with seizures at times. These may follow sugar tolerance tests or intravenous glucose administrations. Full or excessive carbohydrate diets had no elevating effects on the sugar curve nor did they bring about a severe drop in the third or fourth hour periods.

High protein diets cause little or no change in the tolerance in the normal while high fat diets have a marked lowering effect. With the latter point in view high fats have been used clinically in spontaneous hypoglycemia with some success. Some authors claim relief from attacks and an improvement in the blood sugar values while others found temporary relief with no marked effect on the blood sugar curve. Ziskind (3) however, was unable to show any effect on the blood sugar curve following the ingestion of 100 grams of fat. Weeks and others (2) found a marked diabetic response to very high fat diets in non-diabetics. One case receiving 322 gm. of fat a day showed a blood sugar peak of 0.357 at the post prandial rise. We were unable to note any effect on the blood sugar curve of this patient from the use of an identical diet.

All the various endocrine products tried, with the exception of excessive doses of adrenalin, were unable likewise to produce any recognizable change in the blood sugar values. Insulin was given for two periods. At first it was injected in gradually increasing doses to determine whether or not the patient was unusually sensitive to its action. Although hypersensitiveness has been reported frequently in hypoglycemia, we found the reaction normal in this case. The second time it was tried it was given following the suggestion of John (4) who found a return to normal blood sugars with its use in spontaneous hypoglycemia. Furthermore he noted a continuance of normal blood sugars in his patients for a considerable period following the discontinuance of the insulin. In our patient there was no change in the blood sugar values after a week of moderate daily doses. In an investigation of the 3 cases reported by John we found 2 to have been known as diabetics. The response to the insulin treatment may be due, in these cases at least, to a pancreatic dysfunction.

Thyroid extract had no effect on the blood sugar curve. In the usual case of hypothyroidism or myxedema two grains of thyroid extract a day keeps the patient in good condition. We gradually raised the thyroid dose in our case to 27 grains a day and this was continued for over 5 weeks without causing any change in the blood sugar levels. There was a rise in the pulse rate to 130 during that period while there was no change in the B.M.R. For the next 14 weeks the patient received a daily dosage of 15 grains of thyroid extract. The long continued thyroid therapy was carried on because of the possibility of complementary action of the thyroid with the other endocrine products. According to F. M. Allen (5) the thyroid is not diabetogenic in its action beyond intoxication and stimulation of total metabolism. There was no evidence that the thyroid extract assisted any other endocrine products in our patient.

Extracts of the pituitary were of no value in raising the blood sugar. Pituitrin,

the extract of the posterior lobe, ordinarily considered a potent diabetogenic factor, was given to the clinical limit without effect. This lack of effect was first noted by Cushing in 1912 and has been corroborated by others since. Anterior pituitary extract by mouth was given up to 50 grains a day without effect. Likewise anterior extract, parenterally, failed to produce any results. This is suggestive of insufficient dosage, inert extracts or lack of sensitiveness to the effect of pituitary extracts. Our feeling toward this is that all three factors are involved. Failure of the pituitary to act makes it quite positive that cases of chronic hypoglycemia have lost their normal sensitiveness to posterior pituitary influence. Furthermore, the oral form of commercial anterior pituitary extract is generally held to be inert and animal experimentation shows that it requires large doses of freshly prepared solution to produce any effect on the blood sugar values.

There was no response to the parenteral injection of anterior pituitary-like hormones with moderate doses over a reasonable period and the Aschheim-Zondek test showed that there was no loss of this anterior pituitary-like hormone in the urine.

Extracts of the adrenal failed to produce the normal blood sugar response. The commercial oral product seemed inert. Adrenalin however, when given in excessively large doses caused a delayed marked rise in the blood sugar. Lack of reaction to normal doses of adrenalin is a common finding in chronic and spontaneous hypoglycemia. Nevertheless, Feinier and others (6) noted a normal response in two severe cases of proven adenoma of the pancreas.

This very commonly found poor response to adrenalin has been generally explained as a lack of glycogen storage in the liver. There has also been suggested an exhaustion of the body adrenalin. The third possibility is that the adrenalin has lost its ability to release the liver glycogen. There is great difficulty in getting a correct estimation of the liver glycogen under these circumstances. Retention of glycogen in the liver as found in Von Gierke's disease has never been reported in this connection. Wilder (7) in his classical case of hypoglycemia and hyperinsulinism from an adenocarcinoma of the islands of Langerhans, found the liver "gorged" with glycogen.

A lack of secretion by the adrenals does not seem at first to be compatible with the slightly lowered blood pressure and lack of asthenia in this case. Nevertheless the fact that it took 4 to 5 times the normal dose of injected adrenalin to produce systemic effects as well as blood sugar changes points to a need of more adrenalin in the blood. On the other hand if one does not feel that less than the normal amount of adrenalin was secreted by this patient he may bring suspicion on some of the other diabetogenic endocrine products. It is highly possible that a deficiency of one of these may automatically increase the need for more adrenalin in the system. Having accepted this case as one of adiposo-genital dystrophy we can attribute it to a pituitary deficiency, more than likely of the anterior portion of the gland. Hence we have reasonable evidence of the absence of the normal stimulus of a commonly accepted diabetogenic acting principle. We cannot say that the adrenalin has lost its ability to release the liver glycogen since it does that when the dose is raised.

The mechanism by which the glycogen is released from the liver is not yet clear. Cannon (8) believes that the liver cells are not directly subject to sympathetic control but respond to adrenalin from the adrenal medulla and also to secretin. As he points out histologists have not been able to demonstrate any nerve ends in the liver cells. F. M. Allen (5) feels that adrenalin has one specific function, namely the augmentation of sympathetic activity, and that a mobilization of glycogen is merely incidental to this function.

One might explain the failure of adrenalin and pituitrin to raise the blood sugar by the liver cells being refractory to the hormonal action of these secretions. On the

other hand if adrenalin alone stimulates glycogenolysis and pituitrin produces the action by its effect on the adrenal glands there may be a lowered sensitivity of the sympathetico-adrenal system. The apparent cause in this case would be a lowered sensitivity of the pituitary and a lowered secretion of the adenotropic AP principle.

Other substances, such as the anterior pituitary-like hormone of the urine, testosterone propionate, benzedrine sulphate and ammonium chloride, gave no evidence of having any effect on the height of this patient's blood sugar.

The results of a number of diversified blood sugar tests that were tried are of interest. As was noted before, the blood sugar response to a meal or to 100 gm. of glucose was abnormal. With fasting blood sugars consistently in the low normal or hypoglycemic range there was a poor blood sugar response to a meal or a standard glucose tolerance test. In each instance there was a rapid return to the fasting value. The tolerance test curves might be called low plateau in form.

A tolerance test with a larger glucose meal (250 gm.), an extra estimation after 15 minutes and an extension to 4½ hours gave a curve much the same as with the 100 gm. There was no reason to believe that large doses of glucose overwhelmed the sugar metabolism or that they created a state of hyperinsulinism. The 15-minute reading showed that the peak of the curve occurred near the ½-hour mark, which is normal for the high point. There was, however, a urinary spill of one plus at the end of the first hour with a trace at the 1½, 2 and 2½-hour periods. This shows the inflexibility of this patient's blood sugar curve with a spill over the threshold rather than a rise to even average values.

Alimentary sugar tolerance tests were started with 150 gm. of glucose by mouth. It was found that the first spill in the urine occurred following the ingestion of 250 gm. of the glucose. This would indicate a carbohydrate tolerance considerably above normal but still less than the findings of Cushing in pronounced cases of pituitary insufficiency.

It was interesting to note the type of curve obtained during a starvation period. After 16 hours of fasting a blood sugar curve was determined for 3½ hours. The 6 blood sugar readings were approximately the same giving a flat curve. There was no indication of a sharp drop towards the end of the curve as is often seen in spontaneous hypoglycemia. There was, instead, a noticeable effort on the part of the sugar metabolism to keep the blood sugar running along at the same value.

In connection with the last finding it can be seen that while this patient had blood sugar values much below the average and many in the arbitrary hypoglycemic range, he did not, however, at any time reach the low levels commonly found in the cases of hyperinsulinism proven at operation.

The standard Rose-Exton sugar tolerance test gave a diabetic response to the second dose of glucose. One infers from this that there was less insulin stimulation than normal from the initial glucose. When the glucose dose was doubled (200 gm.) there was more evidence of a diabetic response. A prolongation of the test showed, however, a quick return to about fasting value and a tendency to stay at this figure. When the standard Rose-Exton test was performed by giving the glucose by vein the ½-hour value was only slightly above the sugar tolerance tests made orally. There was no rise in the blood sugar values following the second dose of glucose, in fact there was a considerable drop. When continued out for two hours there was evidence of a return to the fasting value. The normal curve proposed by Fairley (9) for the intravenous test was: after 10 minutes, 344; one hour, 137; 1½ hours, 93; two hours, 84. This curve, it must be remembered, represents only one dose of glucose given after a fast. In our case there was decidedly less of a rise following the

glucose injection. There was also a spill of 2 plus sugar at the ½-hour mark and and also at the 1-hour period.

Several inferences are possible from the blood sugar response to the intravenous route. There is more corroboration that the blood sugar mechanism active in this patient is adverse to having the blood sugar rise above 133 at any time. It can also be seen here, as was noted previously when 250 gm. of glucose was given orally, that there was a urinary spill with the blood sugar values of 125 mg. and less.

SUMMARY

Studies of the blood sugar values in a case of adiposo-genital dystrophy with chronic hypoglycemia have been presented. We were unable to raise the prevailing subnormal blood sugar values by any form of diet. We were also unable to produce normal blood sugar values by the use of such commercial endocrine products as oral and parenteral anterior pituitary extracts, pituitrin, anterior pituitary-like hormones, insulin, testosterone propionate and oral extracts of the thyroid and suprarenal glands. Likewise there was no change following the use of ammonium chloride or benzedrine sulphate. There was, however, a poor response to the average dose of adrenalin and a delayed marked rise following the use of excessively large doses.

The sugar tolerance test results were shown to be almost consistently low plateau in form. Larger glucose meals gave practically the same response both in the standard and in the extra high glucose variation. When the glucose was given by vein there was a very poor response at the ½-hour mark and a second injection at this time produced a sudden drop in the curve. A starvation blood sugar curve continued to the 19th hour gave a plateau response. An alimentary glucose tolerance test showed a threshold raised to accept almost 250 gm. of glucose by mouth. A sugar tolerance curve following the use of 250 mg. of glucose indicated that a spill occurred when the blood sugar reached the height of 116 mg. An intravenous Rose-Exton test showed a marked spill over the threshold with the blood at 125 mg.

We can conclude then, that the blood sugar values in this case are very stable and only found to respond to excessive doses of adrenalin. We can also conclude that there is a noticeable tendency on the part of the sugar metabolism to keep the blood sugar at subnormal values even to the extent of causing a spill of sugar into the urine at those values.

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SIMMONDS' CACHEXIA

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SIMMONDS' CACHEXIA is a disease of the pituitary gland, occurring usually in women, and characterized chiefly by progressive asthenia, cachexia, anorexia, amenorrhea, premature aging and a low basal metabolic rate.

The major signs and symptoms of Simmonds' disease are easily understood even from our present incomplete knowledge of the anterior pituitary hormones. There is moderate to complete hypofunction of the anterior lobe of the pituitary, resulting in the decrease or absence of the gonadotropic secretion, of the growth, fat metabolism, lactogenic, thyrotropic, adrenotropic, parathyrotropic and diabetogenic hormones. As a result, the patient will present to some degree the combined signs of hypothyroidism, hypogonadism, hypoadrenalsim (cortical) and hyperinsulinism.

The emaciation is the result not only of anorexia, but also of disturbed fat metabolism. From the secondary hypothyroidism ensues the low B.M.R., the coarse dry skin, the loss of hair and nails, and probably many of the mental signs, especially depression.

The hypoadrenalism produces mild pigmentation, hypotension and probably gastrointestinal disturbances. The decreased salt elimination not infrequently observed (1, 2, 3) is possibly related to the hypocortical factor.

In the female the hypoovarianism is responsible for the amenorrhea, the genital and breast atrophy, and the decreased or absent libido. In the male (4, 5) failure of spermatogenesis, and atrophy of the testicles, prostate and seminal vesicles is seen. In both sexes the loss of pubic hair may be observed.

The absence of the diabetogenic or antiinsulin factor results in a hypoglycemia and a sensitivity to insulin. The glucose tolerance curve is low, as in a case of hyperinsulinism, and recurrent attacks of hypoglycemia may produce symptoms simulating hysteria (6). In a number of autopsies hypertrophy and hyperplasia of the islet cells of the pancreas have been observed (1, 7, 8). The senile appearance of the patient may be attributed to the general hypometabolism, emaciation and atrophy of the skin and its appendages, the latter probably the result of the absence of the growth hormone.

Other signs and symptoms consist of a hypochlorhydria, anemia, eosinophilia (rarer than generally known), vague epigastric pains and attacks of vomiting, scattered paresthesias, headache, a diminished specific dynamic action of proteins (9), vague joint pains, edema of the legs (8), bouts of extreme thirst, somnolence, hypothermia and bradycardia. Decreased sensitivity to adrenalin is common. Orthostatic hypotension has been seen in a number of cases (10, 11). Alterations in the roentgen appearance of the sella turcica are uncommon. Mental symptoms of all kinds and degrees are seen in Simmonds' syndrome, and may dominate the picture. Cahane (12) reports a case of typical melancholia in a 43-year-old woman with vague signs of a Simmonds' syndrome, who recovered completely and rapidly under anterior pituitary therapy.

The following two case reports from the service of one of us are representative of two types of Simmonds' disease. *Case 1*, M. R., a female, age 46, white, American (fig. 1), was admitted to the Jewish Hospital Oct. 30, 1934, with a history of gradually increasing weakness, fatigue, and an increasing anorexia of 7 months' duration. She had lost 30 lb. in this time. Frequent vomiting, but no abdominal pain or dyspepsia had been present.

Systemic review revealed occasional mild headaches together with tinnitus in the left ear. Her menopause had occurred 2 years previously. Some palpitation and dyspnea on exertion had been noted. She had had 2 miscarriages and 1 living child. Her father died of tuberculosis.

On physical examination, the patient appeared underweight and suggestively presenile. The tonsils were hypertrophied and infected. The blood pressure was 110/60. The abdomen, chest, heart, pelvis and extremities were essentially normal. Her weight was 85 lb.

Gastric analysis showed a complete achlorhydria, even with histamine stimulation. The chest and gastrointestinal x-ray studies showed normal findings, except for ptosis of the transverse colon. An electrocardiogram revealed mild depression of cardiac function. Kidney function tests were in the low normal, her urine persistently revealing occasional hyaline casts and white blood cells. The blood cell count was normal, 5,000,000. Biliary drainage was normal; serology test results were negative, and her blood chemistry analysis results were normal, the sugar being 88 mg.%. She refused to remain in the hospital after a 12-day stay and was discharged with a diagnosis of achlorhydria of unknown origin.

At home she continued to decline steadily. She had complete anorexia, became very pale, and her weakness confined her to bed. She began to notice occasional tingling of the extremities. On April 9, 1935 less than 6 months after the first hospitalization, she was readmitted to the wards. At this time she was emaciated, cachectic, pale, and appeared to be at least 75 years old. Temperature was subnormal and blood pressure was 88/62. Nothing significant was found on physical examination. No skin or mucous membrane pigmentation was noted.

A flat plate of the abdomen was negative; a complete achlorhydria was again noted. X-ray examination of the skull revealed a normal pituitary fossa. The urine contained hyaline casts with clumps of white blood cells. Hemoglobin, red blood cells and white blood cells were 74%, 4,200,000 and 9700 respectively with 60% polys, 40% lymphocytes. The blood sugar was 60 mg.%, plasma chlorides were 540 mg.%. The blood cholesterol was 150 mg.%. No glucose tolerance or B. M. R. determined because of the condition of the patient.

During her stay, she ran a persistent subnormal temperature. On April 16 after 5 days of 5 U insulin therapy the patient suddenly developed a circulatory collapse, from which she apparently was recovering, but on April 17 she suddenly died. A diagnosis of Simmonds' cachexia was made.

Postmortem examination showed considerable emaciation of the body. The features were drawn, the cheeks wasted, the skin over the entire body, dry. The general configuration of the body was within normal limits, but the given age of the patient, which is 47, corresponds poorly with the marked senile appearance, which would seem to be 60 or 70 years. The hair was grey; the body hair had largely disappeared; the eyebrows were sparse. The lower jaw presented the atrophy usually associated with high age levels. The breasts were atrophied but equal in size and consistency.

The fat in the panniculus and in the normal sites of deposit was scanty. The muscles appeared to be fairly well preserved. There was no fluid collection in any of the serous sacs.

The heart was small even for the body size, weighing 120 gm. The shape was normal. The pericardium was smooth. The myocardium was of fair tonus, but there had been probably considerable atrophy as indicated by the tortuous course of the large coronary vessels. There was no gross scarring in the myocardium and the vessels were patulous and showed little or no sclerosis. The left ventricular wall was 9 mm. thick, the right ventricular wall, 2 mm. in the mid portions. The valves were normal. The aorta was markedly free of sclerosis throughout.

The lungs showed evidence of an old catarrhal bronchitis but no pneumonia or any other inflammation of the lung parenchyma. The spleen weighed 100 gm. and was of normal consistency. The left adrenal appeared to be entirely missing. The periaortic lymph nodes, however, were moderately enlarged. In the area of the sympathetic plexus over the adrenals there was a small oval flattened greyish piece of tissue, 2.5 × 1.5 × 2 cm. On section this was composed of greyish tissue, possibly only lymphoid, although it resembled accessary chromaffin tissue. The right adrenal was not recognized as such, but in its place were a small number of perioartic lymph nodes, one of which on section was firm, white at the periphery, and possibly cortical tissue. In its center it had a longitudinal reddish zone, possibly the medulla of a sclerosed adrenal. Each kidney weighed 100 gm. and was normal in all respects. The liver weighed 1000 gm. and presented a normal appearance and consistency. The gall bladder and large bile ducts were normal. The pancreas was of normal length, but thin, and suggestive of some degree of atrophy. There was no gross fibrosis. The gastrointestinal tract showed: *a*)

normal esophagus; *b*) moderately dilated stomach in which the rugae were largely gone with numerous small white nodules in the mucosa and submucosa, most plentiful in the cardiac end and fundus and gradually disappearing toward the pylorus. There were small points of recent mucosal hemorrhage. The intestinal tract showed nothing of note.

The uterus was small and fibrotic. Both ovaries were very sclerotic and small, and showed little or no evidence of functional activity. The thyroid was rather small, weighing less than 10 gm. On section it showed a whitish pallor and a consistency suggestive of fatty infiltration. No colloid could be seen grossly. The brain was of normal contour and consistency, the basal vessels were smooth. The pituitary was grossly normal, rather firm, and fibrous.

Histology. There were two types of sections from the adrenal areas; one type was from the large ganglion removed from the left side and showed characteristic ganglionic and nerve tissue. No sign of adrenal medullary structure was seen in repeated sections until one small section was reached which grossly was about 4 mm. long by about 1.5 mm. wide and apparently a part of the ganglion structure above mentioned. This showed atypical medullary tissue. It was surrounded by loose and vascular supporting tissues in which were numerous round cells, apparently lymphocytes. There was nothing that would suggest the presence of any cortical remnant and one must conclude that this is a residual or compensatory chromaffin development in the sympathetic plexus.

The sections from the right adrenal site showed nothing but a marked lymph-glandular structure in which there was distinct hyperplasia not malignant but nevertheless atypical in the irregular distribution and hyperplasia of the germinal centers. In another section from the same area a few isolated, very large cells, containing relatively small nuclei and having a tendency to develop in small columns or rosettes and filled with an eosin-staining granular cytoplasm, may possibly be cortical cells.

Section of the stomach showed a marked atrophy of the glandular structure, a heavy infiltration of lymphoid cells and a muscularis that appeared fairly thick because of edema, but in which individual fibers appear to be distinctly atrophied. Another section from the fundus of the stomach showed the same thing with an attempt at regeneration of the tubular elements of the mucosa. The musculature change was striking. There was distinct muscle atrophy. A section from the cardiac end of the stomach showed a striking degree of atrophy of the mucosa and of the muscularis and greater lymphoid hyperplasia in the mucosal layer. Another section from the cardiac end of the stomach showed a comparatively thick muscular layer, thickened by edema; cystic degeneration of the mucosa, atrophy, and a squamous epithelial layer continued in the termination of the mucosa. This section apparently came from the esophageal cardiac junction. The muscle portion both here and at the pyloric sphincter appeared to be out of proportion to that seen in the muscle of the stomach proper. Another section from the pylorus showed relatively very thick musculature and moderate atrophy of the mucosa with some marked regeneration of the tubular epithelium.

The liver exhibited marked vacuolization of the cytoplasm. Many cells consisted of nothing more than the nucleus and the cell wall. The nuclei themselves showed various degrees of bionecrotic change. The emptying of the cytoplasm content was striking in many areas, and is similar to that which has been described in experimental hypoglycemia.

In the thyroid gland few acini were present; containing scarcely any colloid. There was no hyperplasia despite the absence of colloid. Most of the glandular tissue had been replaced by lymphatic tissue and fibrosis, especially by lymphoid hyperplasia. In other places the acini had undergone depression atrophy because of the lymphoid infiltration. Another section of thyroid showed substantially the same marked atrophy with lymphoid replacement and overgrowth of connective tissue.

In the pituitary mild interstitial fibrosis was seen in most portions of the gland, in addition to which there were two gross scarrings that traversed the length of the anterior lobe. The cells were mostly all chromphobic. A few basophilic cells were present, but no eosinophiles were seen. The anterior lobe was studied in sub-serial section and the above findings were the same throughout. The posterior lobe and the pars intermedia showed nothing of note.

Case 2, L. R. (fig. 2, 3, 4), a white unmarried female, age 24, was admitted to the wards of the Jewish Hospital on April 15, 1935, complaining of weight loss, weakness, constipation, anorexia, vomiting. History of illness dated back to 1932 when she was forced to break off her

Fig. 1. *M. R.* Age 47, April 9, 1935. Fig. 2. *L. R.* Age 17, 1928, before onset of illness. Fig. 3, *L. R.* Age 24, May 6, 1935, during first hospitalization. Fig. 4. *L. R.* Age 28, September 16, 1939. after treatment.

friendship with a young man of whom she was fond, because her parents objected to him. The man soon afterwards married, but persisted in repeatedly informing the patient how unhappy he was with his wife.

Soon after this incident, the patient began vomiting almost daily after meals. She also became very constipated, and although medication relieved her for a while, in 1933 she again began to vomit. At the time of admission, she was able to retain no food.

After 1933, she gradually became more lethargic, and experienced increasing fatigue. Her previously constant weight of 110 lb. had now diminished to 72 lb. She was so severely obstipated that weeks would pass between bowel movements.

During the period immediately preceding hospitalization she began to complain of chilly and cramping sensations in the abdomen. In addition, she was continually thirsty, drinking 8 to 10 glasses of water daily. She also developed hot flushes of the face.

Her menses, previously regular, became irregular at the start of the illness, with a history of amenorrhea of 11 months in 1932. Since July 1934 there had been a complete amenorrhea during which period she suffered from depression, palpitation, melancholia and a fear of some impending calamity. Her past medical history was unimportant. She smoked moderately, and worked occasionally in her father's store.

Physical examination showed an anemic-looking young woman with atrophic breasts and generally wasted body. Her blood pressure was 95/70; pulse, 60. The biceps and triceps reflexes were exaggerated. Patellar and achilles reflexes were diminished.

On April 24, an injection of 3 minims of adrenalin was given hypodermically to discover its effect on blood pressure, pulse rate and temperature. No effect was noted after 10 minutes.

X-ray studies of the skull, vertebra, femora and pelvis gave essentially normal findings. The gastric analysis showed slight hypoacidity. Perimetric eye studies were normal, and basal metabolic rates were −28% on two occasions. Glucose tolerance tests were as follows: fasting, 63 mg.%; ½ hour 126 mg.%; one hour 169 mg.%; two hours, 162 mg.%. Blood calcium was 9.0 blood phosphorus 3.6, and blood cholesterol, 192 mg.%. Urinalysis result was normal. A urinary hormone assay on May 5 revealed complete absence of APL hormone with an overabundance of theelin. Hemoglobin was 90%, red blood cells 4.9 million, white blood cells 9400 with 64% polys and 36% lymphocytes.

Her weight at this time was 73 lb. A diganosis of pituitary cachexia was made and the patient was given APL extract parenterally, 200 R.U. 3 times weekly, and referred on May 6, 1935 to her attending physician. On discharge the patient weighed 70½ lb. The patient continued to receive tri-weekly injections of APL substance plus small doses of thyroid substance.

On June 11 a hormone assay of her urine revealed 10 to 15 U of APL hormone and about 75 M.U. of estrogens, per 24-hour specimen. Her general condition improved, but her weight did not increase until the thyroid substance was discontinued. She then steadily gained in weight with a marked general improvement. On Sept. 18, 1935 the patient developed a full menstrual flow for the first time in 16 months. Her weight then was 96 lb. She was cheerful, happy, and for the first time felt that she was going to get well. Following her menstrual flow, APL substance was reduced to 450 R.U. per week. Her menses continued for one week. Her weight gradually increased. On the 16th of October, 1935, she again had a normal menstrual flow. At this time in addition to APL substance she was also given hypodermic tonic doses of sodium cacodylate. Her B.M.R. was now +6 in contradistinction to her previous B.M.R. of −28. It is interesting at this time to note that improvement not only continued in her mental symptoms but that there was a marked change in her secondary sexual characteristics. Two months after return of menses her breasts had regained their original size. Following this improvement she became irregular in her visits to the physician's office. A hormone assay made at this time of her premenstrual urine, showed a normal theelin content, with the absence of APL substance (which is normal for premenstrual urine). Her weight was 111 lb. Her general condition was excellent, except that she still continued to have gastrointestinal disturbances, such as postprandial nausea and vomiting. She returned some 3 months later with a history that her menses had become somewhat scanty. Pituitary treatment was instituted and 2 months later she menstruated. The patient failed to return for treatment for about 6 months, at which time she again became amenorrheic. Her weight which had previously been 111 lb. was now 91 lb. with a return of all of the previous symptoms. She was readmitted to the hospital on Feb. 21, 1938. Her weight was 82½ lb., her blood pressure 84/50, with a symptomatology essentially the same as on her first admission. She had not menstruated since January 1938. Her blood chloride level was 380 mg. Her response to adrenalin was again minimal Five units of insulin sent her blood sugar down from 75 mg.% to 48 mg.% in one hour. Her eye grounds were normal. Urinary studies revealed a complete absence of estrogens and APL hormone. X-ray on February 23, revealed a fairly small pituitary fossa. A flat plate of the abdomen was negative. The gastric analysis result was again essentially normal. Her red blood cells varied from 3.8 to 4.3 million, white blood cells 6500 to 7600 with normal differential counts. The blood cholesterol was 215 mg. and the B.M.R. was −15.

The patient was given 300 R.U. of APL substance 3 times weekly, beginning on March 8, 1938. By April 5, 1938, the day of her discharge, she felt much improved, and had gained 12½ lb., and the vomiting had ceased. On May 1, 1938 she had a normal menstrual flow. She was referred to a convalescent home for further care. The injections of pituitary substance were continued and on June 1, 1938 and subsequent months her menses had been regular. Her weight had increased to 103 ¾ lb. At the present time the patient has not received any treatment for about six months and apparently is continuing to do well, has a normal menstrual flow, and her general symptoms are improved.

DISCUSSION

Etiology. Silver (13), found atrophy and fibrosis of the anterior pituitary in 26 of 39 autopsies of cases of Simmonds' disease. He thought these were due to previous embolism particularly as result of puerperal sepsis. In the other 13 cases new growths destroying the anterior lobe of the pituitary were found, 5 cysts, 2 basophilic adenomas, 2 carcinomas, 2 gummas, 1 tuberculoma, 1 endothelioma. This observer stated that occasionally an atrophic pituitary was found without clinical symptoms.

Riecker and Curtis, in 24 cases with autopsies, found partial or complete destruction of the anterior lobe in all cases, 14 allegedly due to puerperal sepsis, 5 due to syphilis, 4 due to cysts, 2 to tuberculosis and 2 of unknown etiology.

Wolf (15) believes the disease is an important sequel of Fröhlich's syndrome, although practically all the recent cases do not substantiate this view. Pathologically, he states that most commonly there is an atrophy of the anterior lobe of the pituitary due to an obstruction of the blood supply from embolism in puerperal sepsis. Tuberculosis, syphilis, actinomycosis, trauma, tumors, cysts and pituitary inflammations are less frequent etiological agents. Wolf believes that mild cases with only transitory symptoms are not uncommon, and hypothesizes a temporary edema of the pituitary to explain such occurrences.

Apparently all observers agree that childbirth is one of the commonest contributing factors, and until recently sepsis was considered important. In 1914, Maresch (16) first called attention to the fact that a number of cases have followed repeated labors, especially when accompanied by hemorrhage. These he believed were produced by a functional exhaustion of the pituitary. Herrick (17) also concludes that such a functional exhaustion is an important factor, since the pituitary gland increases in size during pregnancy and returns to normal during the puerperium, at which time Simmonds' disease is most apt to appear.

May and Robert (18) also favor this functional exhaustion theory. Sheehan and Murdock (19) have greatly clarified the rôle of parturition in pituitary dysfunctions. They showed that extensive ischemic necrosis of the anterior pituitary is not an uncommon incidental autopsy finding in women, who die in the puerperium. In all cases the patients collapsed during delivery, usually of severe hemorrhage, although necrosis is not seen histologically less than 14 hours postpartum. In 46 cases of women dying 14 hours or later after delivery, 13 showed anterior pituitary necrosis. Moreover, in cases of Simmonds' disease dating from a previous delivery, the pathology findings in the anterior pituitary correspond to the healed stage of the above mentioned ischemic necrosis. Sheehan therefore is quite confident that sepsis plays a very minor rôle. Encouraged by these observations he decided to search for evidence of hypopituitarism in patients who survived collapse and hemorrhage during delivery. In a follow-up study of 128 women who had had hemorrhage collapse during delivery, he found (*a*), absence of mammary reaction after delivery, (*b*), menses absent or infrequent, (*c*), hypersensitivity to cold, asthenia, apathy, loss of libido, (*d*), emaciation (2 patients developed frank Simmonds' disease), (*e*), premature aging, (*f*), genital atrophy. To enhance the significance of these findings, it must be added that the more severe the hemorrhage, the greater the number and degree of these hypopituitary findings. Moreover, they were absent in the normal group.

From this important contribution, it becomes apparent that hemorrhage collapse during labor or puerperium often results in ischemic necrosis of the anterior pituitary, which may be severe enough to cause immediate death or a later severe Simmonds' syndrome, or so small as to cause only an absence of the mammary reaction with, of course, many intermediate types.

The increasing frequency of hemorrhage and collapse common with increasing parity and age therefore explains the occurrence of Simmond's disease in old multipara. Indeed, recently many cases of Simmonds' disease have been observed in women who had hemorrhage collapse during delivery (1, 2, 9, 14, 20–24). Sheehan collected from the literature 34 such clinical cases. Only 25 had a history of hemorrhage during delivery, but he believes their records were inadequate on this point.

In our *case 1*, the autopsy revealed an almost complete absence of eosinophilic cells in the anterior pituitary. This finding is of great interest. Brown (25) mentions a number of necropsies of Simmonds' syndrome in which only eosinophilic cells were destroyed or absent. Nevertheless it seems possible that Simmonds' syndrome can be due entirely to a functional depression of the anterior pituitary.

Pathological picture. Autopsy findings explain the effect of absent anterior pituitary secretions on the viscera. In advanced cases, microsplanchnia is the rule. Even in early cases gross and microscopic atrophy of the thyroid, gonads, adrenal cortex and parathyroids is seen (1, 2, 4, 5, 7–9, 13, 15, 23, 24, 26). Our case presented very similar findings at autopsy.

This case is a fairly typical example of classical Simmonds' disease. She had had 3 pregnancies, and menopause at 44 years of age. Her prominent initial symptoms were weakness, anorexia, great weight loss, vomiting and dyspnea. Initially her blood pressure was 110/60. She had a complete achlorhydria.

She declined steadily, losing weight, and becoming cachectic and senile in appearance. The temperature became subnormal, and her blood pressure dropped to 88/62, and blood sugar declined to 60. She died in circulatory collapse, possibly as an unrecognized hypoglycemic reaction, which is commonly seen in these cases. Unfortunately, neither a glucose tolerance nor a B.M.R. was performed.

At autopsy, microsplanchnia, absent right and atrophic left adrenal gland atrophic ovaries and thyroid gland were found. Grossly the pituitary seemed normal but histologically an almost complete absence of eosinophils was noted.

Functional Simmonds' syndrome. It is our belief that the pituitary secretion is subject to functional suppression in certain individuals, and can therefore give rise to a chain of symptoms indistinguishable from true Simmonds' disease, or if a milder suppression exists, can merely result in a thin, undernourished individual. As a rule, these cases are more or less easily controlled by substitution therapy, and rarely come to postmortem. They exist commonly in postpubertal females, and are not infrequently diagnosed as anorexia nervosa. It was apparently this type of case that Silver, in his review of the literature, rejected as true Simmonds' disease, since autopsies were not available.

As a criterion of this type of case we stipulate that the patient be a young nulliparous postpubertal female, with all the classical signs of Simmonds' disease, who responds well to substitution therapy. Many cases of this type are present in the literature (18, 27–35). It might be argued that such cases are in reality anorexia nervosa (since a psychic onset is not uncommon) and that the fine response is merely a psychic response to injections. The response of some of these cases to psychotherapy and high caloric and carbohydrate diets is usually considered sufficient proof of their true nature. However, Mahoney (36) has shown that in experimental hypophysectomy, the cachexia can be prevented by a high carbohydrate diet.

Pituitary hypofunction can occur without extensive destructive pathology in the gland. Plummer and Jaeger (5) report a case of Simmonds' disease in a male, at whose autopsy a glioma was found pressing on the pituitary, which appeared normal grossly and showed edema histologically. Samet-Mandels (37) reports a somewhat similar tumor case in which the pituitary, though pressed upon, was grossly

and microscopically normal. DeLaure and DeGennes (6) presented typical Simmonds' disease in a nulliparous girl who died in two years after onset, at whose autopsy typical visceral findings of the disease were seen, but whose hypophysis was completely normal. All these authors concur in the belief that the pituitary can be completely inactive functionally, though normal in gross and histological appearance. It is not unreasonable to assume that a functionally inactive pituitary will undergo regression in time so that the atrophy of the anterior pituitary found in longer standing cases is possibly the end result of a once functional hyposecretion. A vague clinical entity with pluriglandular sclerosis has been reported in the literature in which a clinical Simmonds' syndrome exists, but pathologically insufficient damage of the pituitary is found to be wholly explanatory. This type of case could possibly represent a functional hypopituitary disease in which the atrophy of the other endocrine glands is secondary to the lack of pituitary secretion.

In our second case the onset of symptoms began at the age of 22, after a definite psychic shock. Her signs and symptoms were typical of Simmonds' syndrome and her response to APL substance was striking and uniform. Lapse in treatment produced a relapse, while renewal of treatment produced a remission. Even so, such a case might be termed (by many writers) an anorexia nervosa. However the complete absence of anterior pituitary hormone and estrin in her urine suggests a hypopituitary etiology. Kunstadter (29) and Escamilla and Lisser (9) each report a case in which these studies were performed, each having absent estrin and gonadotropic hormones. This may be a useful diagnostic test, but unfortunately is rarely performed. Another test, described by Aron (38) consists of injecting 5 cc. of fresh urine on 3 consecutive days into a 3-week-old female rabbit weighing 160 to 200 gm. The ovaries and thyroid of the animals are then examined histologically. Normally, there is evidence of moderate stimulation of these organs. In hypopituitary cases there is no evidence of stimulation, while in hyperpituitary cases marked stimulation results. Using this test, the originators showed that most cases of so-called anorexia nervosa were hypopituitary in origin. However, other workers have not corroborated this test, which depends too largely on subjective interpretation.

All this evidence makes us feel certain that Simmonds' syndrome can be and frequently is of functional origin. Many other contributors have postulated this functional etiology. Engelbach (39) maintained that many cases are due to transitory pituitary dysfunction. Sheehan (25) distinctly differentiates the organic postpartum cases from the functional type occurring in younger girls. Kunstadter (29) believes that the early type of Simmonds' syndrome may be due to a congenital defect, to toxic causes secondary to an infectious disease, or to functional exhaustion from the added strain of puberty. Bickel (11) believes in the light of modern experimental findings, that functional anterior pituitary insufficiency is a clinical entity, of which the Simmonds' syndrome is a severe form. Others (8, 18, 32, 37, 40–44) believe that functional hypopituitarism can often produce Simmonds' syndrome.

Anorexia nervosa. Clinically, this entity can assume the exact picture of Simmonds' disease, but is apparently merely a severe psychoneurosis seen usually in young girls, in which anorexia is the trigger that causes malnutrition, amenorrhea, low B.M.R., hypotension, low blood sugar and sensitivity to insulin. No satisfactory explanation of the low B.M.R., the low blood sugar and the high sugar tolerance has been advanced. Its differential diagnosis from Simmonds' syndrome is often almost impossible. Richardson (45) feels that many of the Simmonds' disease cases are merely anorexia nervosa, but that a diagnosis can only be made from the results of psychotherapy and a high caloric diet. He adds, however, that psychic inanition may have a secondary depressing effect on the anterior pituitary. Augier and

Cossa (43) and Rougeau (34) feel that even anorexia nervosa has a hypopituitary basis, perhaps accentuated by psychic factors. Many others concur in this belief (42, 43, 44). Sheldon (46) conceives a similar mechanism concerned in both types of disease (Simmonds' disease and anorexia nervosa), one actuated by a psychological factor, the other by a physical factor. It is our belief that a differential diagnosis might be established by blood and urinary estrin and pituitary hormone assays.

There is a growing conviction in the literature that mild cases of hypopituitarism are not uncommon, in which constitutional asthenia, thinness, reduced B.M.R., and hypotension are present to so mild a degree that such individuals are hardly recognized as pathological (11, 15, 29–31, 40–42). Twenty-four individuals with malnutrition, sensitivity to insulin, low blood sugar, were treated by Berman (41) with a pituitary extract and excellent results were obtained. Sheehan showed that mild hypopituitary signs were often present in women who had suffered hemorrhage in labor or puerperium.

Much confusion exists in the literature in the field of hypopituitarism. Many consider cases as Simmonds' disease only when there is an extensive destruction or atrophy of the anterior pituitary with fully developed clinical picture and where death ensues (17, 29), while many others designate such cases as Simmonds' disease whether organic or functional.

We feel that Simmonds' syndrome, as a name, should not be restricted to cases where a distinct pathology is found in the anterior pituitary, but should be applied to all such cases whether organic or functional. As an analogy one might cite diabetes. The pancreatic pathology seen in this disease can vary from a severe destruction of the islet cells to the presence of practically no discoverable pathology, and yet since this disease is a clinical and biochemical entity, it receives one name. We feel that this analogy applies in a large measure to Simmonds' syndrome. It is hoped that future hormone studies will produce a definite and specific test for this disease.

Treatment. In 1927 Smith demonstrated that experimental Simmonds' syndrome in rats could be controlled by anterior pituitary extract injections. Clinically, many favorable results have been obtained with substitution therapy. The earlier workers used the APL substance from pregnant urine, though more recent clinicians have been using anterior pituitary extracts of various sorts. Good results with APL substance have been reported (10, 27, 47–54). Excellent results with AP extracts were obtained (14, 18, 20, 21, 28, 29, 32). Baltzan (34) used antuitrin-S favorably. Failures or poor results have been the experience of others (3, 4, 5, 9, 45, 55–59). The majority of reports are favorable.

Bickel (11) treated 21 cases, of which 10 were cured, 3 improved, 2 did well but lapsed treatment, and 4 extremely advanced cases died, and 2 cases could not be followed. He considers the disease essentially curable, but found extreme variations in the potency of various APL preparations. Every product he used therapeutically was tested for potency, so that only highly potent preparations were employed.

Other hormones have been widely employed to supplement the major therapy. Estrogenic substances, thyroid, insulin and cortin have all been tried, but estrogenic substances seem to be the most consistently helpful. Bickel suggests that in severe cases, a beef pituitary gland be transplanted into the individual for temporary aid until the injections become efficacious. Kylin (60) claims that he has successfully treated 38 cases with animal pituitary transplants.

The successful dosages employed were in the vicinity of 100 to 150 R.U. of APL substance daily, or 1 to 2 cc. of potent anterior pituitary preparations daily, with a gradual decreasing frequency of injections as improvement set in. Estrogenic substance, from 10,000 to 50,000 I.U. weekly, has been employed.

SUMMARY

Two cases of Simmonds' disease are reported, one with autopsy findings, the other successfully treated with APL substance. The autopsied case revealed an unusual pituitary pathology, an absence of eosinophilic cells. Simmonds' disease may be due not only to an organic destruction of the anterior pituitary, but also to a functional suppression of the gland. Hormone assays of the urine should always be made to confirm the diagnosis in doubtful cases, especially where anorexia nervosa seems a possible diagnosis. In Simmonds' disease, gonadotropins are apparently absent in the urine. Hypopituitarism may give rise to very mild forms of this disease, often unrecognized as such.

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THE TREATMENT OF OBESITY DUE TO DIETARY INDISCRETION (OVEREATING) WITH BENZEDRINE SULFATE

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THE TREATMENT of obesity has, in the past, been well discussed in many articles. The universally accepted treatment now usually consists of a diet in conjunction with some form of medication. One group claims that obesity is caused purely by overeating; the second group agrees with this statement, but adds an additional endocrine factor.

With the above in mind, one would try the surest and at the same time the safest method of reducing the adipose individual, and thereby possibly restore normal endocrine function. A drug which has gained recent prominence in several other fields and which has proven of some value in the treatment of obesity, in benzedrine sulfate.

In over five hundred cases of obesity, benzedrine sulfate (methylphenethylamine. sulfate), sometimes referred to as amphetamine, and which was over a year ago accepted by the Council of Pharmacy and Chemistry of the American Medical Association, has proved to be the "drug of choice" in obtaining excellent results. In this series of cases in which 47,000 10 mg. tablets were used, the results were gratifying to both the patient and the physician in all respects. There were no complications approaching a serious nature encountered. It was observed that as soon as the patient passed the 'trying period' of the drug, taking from 3 days to a week, there was no trouble in getting the patient to cooperate.

METHOD OF TREATMENT

Only obese patients who gave a history of overeating, picking between meals, or having the bad habit of eating at bedtime, were selected for this series of cases. After a physical examination, including urinalysis, blood count, and basal metabolism in most instances, the patient was then put on a diet containing between 1200 and 1500 calories. This diet consisted of dry cereals; skimmed milk, tea or coffee (without sugar or cream) as a beverage; fresh and stewed fruits; raw and cooked vegetables; one slice of bread with each meal; and the choice of a portion (6 to 8 ounces) of meat, fish, chicken, cottage cheese or 2 eggs daily. The patient was cautioned to limit the intake of fluid (never ice cold) and to make certain of at least a daily, normal evacuation.

Dosage. The patient was then given the first week's medication (the smallest possible dose) and thereafter returned every week for a check-up and new medication.

The stagger system amounted to the withdrawal of the drug at the end of every month or the loss of the first 20 and subsequent 15 lb. whichever came first, and for the ensuing week prescribing some brand of vitamin B, C and G, to make up for any deficiency in the diet caused by the patient's carelessness. After each of these "vitamin periods" the previous week's dosage was again prescribed and then increased the following week. This prevented a not too rapid increase of the benzedrine and permitted the final strength of the drug, one tablet after each meal to be reached in about 3 months instead of 9 weeks.

The amount of weight to be lost at any one course of treatment was calculated by the rule that not more than from one-fifth to one-sixth of the patient's greatest weight should be reduced at any one course of treatment. If this new final weight was then far from the nearest calculated normal chart weight (according to height and age) then the patient was instructed to return in from 4 to 6 months, when again this procedure would be repeated. Thus, by following this rule, there was not a single case in this series that showed any untoward effects from the loss of weight, even though as much as 60 lb. was taken off by one patient in the course of 3 months. If it was necessary for the patient to return in 6 months to lose more weight, the patient was placed on a maintenance diet for this period of time, so that none of the lost weight would be regained.

The average amount of weight lost for the first week amounted to 5 lb., ranging from $17\frac{3}{4}$ to 2.2 lb. The average loss for a course of treatment (3 to 12 weeks) was 3.2 lb. per week. The average loss of weight for the first months was between 15 and 20 lb. The most amount of weight lost in the shortest period of time was *case 19* (*D.C.*), who lost 36 lb. in 6 weeks. The greatest amount of weight lost was 102 lb. by *case 54* (*R.C.*). With the exception of vitamin week there were very few instances when a continuous and sustained loss of weight was not obtained. This was due to the fact that a state or condition of this kind was not permitted and prevented from occurring. At the very beginning, if the expected weight for the corresponding week was not lost, the patient was asked to recall all the food consumed the previous week (breakfast, lunch and dinner). In this manner many an erring patient was shown the mistakes which were responsible for the improper loss of weight. By the continuous use of this rigid method, which demanded 100% cooperation, there were very few patients who did not, for any one week, lose some weight during the course of treatment.

Table 1, which follows, contains examples of some of the results obtained.

DISCUSSION

In general the following observations were made.

General health. Invariably most of the patients would volunteer such statements as "feeling much better," "had much more pep," "an increased amount of energy making it possible to do much more work than previously," and "catching up with

TABLE 1

Case	Age, sex	Original weight	Final weight	No. of weeks treated	No. of weeks on med.	Total loss of weight	Lost 1st week	Ave. loss of weight	Original B.P.	Final B.P.
		lb.	lb.			lb.	lb.	lb.		
1, M.D.	31 (f)	$120\frac{1}{2}$	113	2	2	$7\frac{1}{2}$	$5\frac{1}{2}$	$3\frac{1}{4}$	128–80	126– 80
2, F.R.	42 (f)	$152\frac{1}{4}$	130	10	8	$22\frac{1}{4}$	5	2.2	130– 90	128– 80
3, P.B.	43 (f)	$152\frac{1}{4}$	$133\frac{3}{4}$	4	4	19	$5\frac{1}{4}$	$4\frac{1}{2}$	130– 84	122– 80
4, P.R.	45 (f)	$193\frac{1}{2}$	141	13	10	$52\frac{1}{2}$	$8\frac{1}{2}$	4	100– 80	128– 80
5, P.M.	28 (f)	$152\frac{1}{2}$	$132\frac{1}{2}$	8	7	20	$5\frac{1}{2}$	$2\frac{1}{2}$	112– 80	122– 80
6, J.H.	18 (f)	$187\frac{1}{2}$	$137\frac{1}{2}$	16	13	50	$6\frac{1}{4}$	3+	106–80	118– 80
7, S.I.	33 (m)	$202\frac{1}{2}$	$171\frac{1}{2}$	5	4	31	$8\frac{1}{2}$	6	90– 60	116– 80
8, T.C.	33 (f)	237	180	15	12	57	10	$3\frac{3}{4}$	118– 80	120– 80
9, F.E.	38 (f)	162	$134\frac{3}{4}$	7	6	$27\frac{1}{4}$	$5\frac{3}{4}$	4	110– 80	116– 80
10, W.E.	46 (f)	$253\frac{1}{2}$	192	15	12	$61\frac{1}{2}$	$10\frac{1}{2}$	4	230–140	158–100
11, H.L.	27 (f)	171	$137\frac{3}{4}$	9	8	$33\frac{1}{4}$	$7\frac{1}{4}$	$3\frac{3}{4}$	110– 80	120– 80
12, R.C.	31 (f)	$152\frac{1}{4}$	126	8	7	$26\frac{1}{4}$	7	$3\frac{1}{4}$	118– 80	130– 80
13, S.G.	42 (f)	206	162	11	9	44	$9\frac{1}{4}$	4	140– 80	128– 80
14, N.H.	33 (f)	$235\frac{1}{4}$	185	14	11	$50\frac{1}{4}$	7	$3\frac{1}{2}$	120– 80	116– 80

TABLE 1—*Continued*

Case	Age, sex	Original weight	Final weight	No. of weeks treated	No. of weeks on med.	Total loss of weight	Loss 1st week	Aver. loss of weight	Original B.P.	Final B.P.
		lb.	lb.			lb.	lb.	lb.		
15, M.W.	44 (m)	295½	225	12	10	70¼	13½	5¾	148– 86	128– 80
16, C.L.	18 (m)	267½	211¾	11	9	56	13¾	5	140–100	120– 80
17, D.P.	33 (m)	244½	194½	9	8	50	11	5½	120– 80	124– 80
18, R.S.	35 (m)	195	154¾	9	7	40¼	9¼	4½	120– 80	126– 80
19, D.C.	56 (m)	180	144	6	5	36	13	6	164– 90	130– 80
20, R.D.	23 (f)	182½	144¼	7	6	36	6½	5	132– 82	122– 80
21, K.D.	22 (f)	168¾	137	10	7	31¾	5½	3	100– 80	116– 80
22, B.J.	22 (m)	204½	176	5	4	28½	10½	5½	124– 82	122– 80
23, B.F.	27 (f)	193¾	160	8	6	33¾	8½	4	145–90	126– 80
24, K.M.	46 (f)	154½	138¼	4	4	16¼	5½	4	146– 90	128– 82
25, A.A.	17 (f)	137½	122	4	4	15½	6½	3¾	110– 70	120– 80
26, A.C.	40 (f)	169	141½	10	8	38¾	2½	3.8	152–100	130– 80
27, B.E.	22 (f)	138½	128½	2	2	10	5¾	5	120– 80	120– 80
28, B.P.	28 (f)	151½	131	6	4	20½	5½	3½	118–80	112– 80
									160– 90	
29, B.H.	26 (f)	194½	156¾	14	12	37¾	5	2¾	140– 90	128– 82
30, B.O.	53 (f)	137	124¼	5	4	12¾	3½	2½	110– 80	124– 80
31, B.J.	46 (m)	193¾	167¼	5	4	26	8¼	5½	140– 80	132– 80
32, B.R.	22 (f)	173½	145	9	8	28½	8½	3+	130– 90	120– 80
33, B.H.	26 (f)	183½	149	7	5	34½	10¼	4¾	120– 80	120– 80
34, B.M.	37 (f)	160¾	138	9	7	22¾	5	3½	130– 82	122– 80
35, B.T.	41 (f)	137	122¼	5	4	14¾	6½	3	120– 80	124– 80
36, B.F.	14 (f)	189¾	143¼	12	9	46½	8	3½	132– 80	114– 80
37, C.E.	43 (f)	242¼	190	11	10	52¼	7¼	4¾	130– 90	128– 80
38, C.R.	46 (f)	164½	136¾	11	9	27¾	6¾	2½	160– 92	130– 80
39, D.R.	32 (f)	235	182¾	11	9	52¼	12¾	4½	150– 90	130– 80
40, D.E.	26 (f)	140	125½	4	4	15	4¼	3½	120– 80	122– 80
41, E.S.	33 (f)	153	131	6	5	22	7	3½	120–100	120– 80
42, E.C.	52 (f)	219½	168	13	10	51½	5½	4	140–110	138– 80
43, E.J.	35 (f)	154	129¾	9	7	24¼	7¼	2¾	110– 80	118– 80
44, F.S.	15 (f)	191¼	154¾	11	9	36½	8¼	3+	130– 80	116– 80
45, T.L.	42 (f)	158½	134	6	5	24½	6½	4	142–100	128– 80
46, F.G.	39 (f)	199½	148¾	13	10	49¾	5¾	3¾	120– 80	120– 80
47, F.R.	19 (f)	167	137¾	9	8	29¼	6¾	3+	120– 80	118– 80
48, W.J.	24 (f)	253	194¾	19	15	58¼	11	3	128– 80	120– 80
49, C.C.	19 (f)	194½	153½	13	11	41	7	3	100– 80	116– 80
50, T.R.	34 (f)	187	143¾	13	11	43¼	8¼	3½	106– 84	110– 80
51, G.M.[1]	37 (m)	248	194¼	10	8	53¾	10½	5.3	120– 80	120– 80
		197½	167	9	7	30½ (81)	7	3¾	130– 80	118– 80
52, N.H.[2]	24 (f)	294	216¾	14	12	77¼	17¼	5½	132– 80	124– 80
		227	200	6	5	27½ (94)	2	4½	120– 80	120– 80
53, P.A.[1]	46 (f)	245¾	187¾	16	12	58	5¾	3¾	128– 80	128– 80
		190¼	163½	8	7	27 (82¼)	6	3½	130– 80	130– 80
54, R.C.[3]	23 (f)	257¼	191¼	15	11	66	7¾	4¼	130–110	114– 80
		189¾	155	11	9	34¾ (102½)	8	3+	128– 82	122– 80
55, F.G.[1]	39 (f)	199½	157	11	9	42½	3½	3¾	120– 80	126– 80
		154¾	139	5	4	15¾ (60½)	5¾	3	128– 80	120– 80

Cases 51 to 55 illustrate the results of a second course of treatment.
[1] Returned 4 months later.
[2] Returned 3 months later.
[3] Returned 5 months later.

duties postponed because no energy or initiative was present before." Some reported their families noticed a favorable change in their temperament. Most explained they felt more "compact" or "solid" although the loss of weight was not proportionate to this compactness or solidness. All agreed that the loss of appetite made dieting easy. Some of the above results were obtained by Rosenthal and Solomon (2). An occasional headache was encountered, especially if ephedrin was being used for some sinus condition. On the other hand, lots of headaches experienced previously disappeared while using benzedrine. Occasionally a divorcee or widow would complain of the increased libido created by drug, while the great majority (all the above series with the exception of 4) stated it had the opposite effect, as reported by Schimmenti (3). In this series it was noted that the benzedrine caused a great deal of pre-menstrual tension, irritability, and a fainting (light-headed) feeling in a good many of the patients for the first menses. It was necessary to warn new patients to expect this pre-menstrual tension, and not to bend down too suddenly. Bad breath and dry mouth were other too frequent complaints. Dysmenorrhea was relieved by benzedrine in only a small percentage of the cases. Several cases of amenorrhea and one case of sterility in this group were cured when the patient attained a normal weight. A few cases of psoriasis, eczema, and acne were either cured or greatly improved during the medication. Likewise, patients going through the menopause were greatly relieved of their bothersome symptoms.

Appetite. To the surprise of many patients who started to reduce, but who really had no intentions of sticking to it, they found that their desire for food had so diminished and the corresponding loss of weight had so thrilled them and given them a sense of well being that they decided to do a conscientious and more serious job.

Blood pressure. With the small graduated doses of benzedrine sulfate employed in our series of cases, one observed that there was a drop in both the systolic and the diastolic readings of high blood pressures to the point where they became normal or almost normal, as formerly observed by Franke (4). Whether these cases of hypertension manifesting a tendency towards normal was directly due to the action of the drug or to the loss of weight per se is a moot question. Several cases of hypotension became normal during the course of treatment. On the other hand normal blood pressures were not affected, or slightly lowered, if any.

Basal metabolism. The records show that there was very little change in the metabolic rate under benzedrine sulfate. At any rate there was very little evidence that benzedrine sulfate caused a loss of weight by increasing the metabolism in the patient, which is similar to the studies made by Lenzi and Lenzi (5). Davidoff & Reifenstein (6) and Donley (7) have all found that the change caused by benzedrine sulfate on the B.M.R. was a slight elevation, most of the time none at all—all of these being temporary.

Urine. As observed with blood pressure readings, there was noted a marked progressive improvement in those urines containing albumin, sugar, hyaline and granular casts, when benzedrine was used to cause a reduction of weight, in conjunction with simple diets. Again the question arises, was it the drug and diet, the diet itself, or the loss of weight that was responsible for these results? At any rate, we know that benzedrine sulfate produces no injurious effects upon the kidneys as ascertained by examinations before, during, and after the completion of a course of treatment with benzedrine sulfate.

Habit formation. In our series of patients there was little evidence of true addiction. True, an occasional patient returned several weeks after the course of treatment and asked for some more of those "pep" pills to restore some of the pep she or he so

pleasantly sensed while taking benzedrine. When informed that these pills were not to be used again, there was not the pleading and insistence which usually is manifested by an individual if a habit forming drug was stopped. However, a warning here is necessary in spite of the following. James (8) writes: "To summarize then, there is so far little evidence of true addiction to benzedrine, and it is improbable that it may be regarded as one of those drugs to which addiction is likely to spread. The reasons for this conclusion are that the unpleasant physical symptoms are sure to show themselves if benzedrine taking is prolonged. Further, repeated resort to benzedrine results in a loss of its stimulating effect in most people." Finally, in addition, Hill (9) quotes, "Recently publicity has given prominence to the fear that some may partake too freely of benzedrine. But Guttman and Sargent (1937) and Wilbur and others (1937) have shown that the ecstasies fade with repetition. It is the unstable neurotic who needs to be kept from the drugs of addiction. These unfortunates, being in discord with life, crave one thing above all others—oblivion. The fact that benzedrine robs them of sleep will quickly make it unpopular."

Gastro-intestinal. In this series of patients benzedrine sulfate in most instances caused a mild constipation which was easily remedied with either a mild laxative or a simple lubricant, as mineral oil. Either one of these assured a daily evacuation. Occasionally a patient complained of the immediate urge to evacuate soon after the taking of the first morning dose. This urge disappeared after several days and constipation set in after several more days. This constipation can be explained by the fact that the benzedrine sulfate was found to relax the smooth muscles of the gastro-intestinal tract and therefore, incidently was found to be of great use for x-ray visualization (10).

SUMMARY

In the treatment of more than 500 cases of obesity with benzedrine sulfate, some of the findings have been reported and the results tabulated. The average loss of weight of 3.2 lb. per week for a course of treatment (3 to 12 weeks) must not mislead one to the injudicious use of the drug or to use it promiscuously in every case of obesity. The reported untoward reactions and the continuous warnings of its possible toxic effects in various journals should be of much significance to both the dispenser and the novice with limited experience who is about to prescribe benzedrine. The patient also must be educated as to the possible danger because of its allergic reactions to some, or because of its cumulative effects. Self medication and overdosing must be eliminated to prevent any future fatalities.

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PREPARATION OF ADRENOTROPIC EXTRACTS AND THEIR ASSAY ON TWO-DAY CHICKS[1]

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IT IS WELL KNOWN that the adrenal cortex shows enlargement and increased function following various experimental procedures and treatments. It is also certain that marked cortical atrophy follows hypophysectomy. Many investigators believe that a specific anterior pituitary (adrenotropic) hormone normally supports cortical tissue and its functions, though Grollman (1), Fazekas (2) and others have questioned the existence of a specific adrenotropic hormone. The first qualitative and quantitative studies in this field were largely based on histological changes in the adrenal cortex. An historical review of the earlier studies was made by Collip (3). Reiss (4) described a specific Sudanophobic zone which appears in adrenals of hypophysectomized rats and which disappears upon treatment with hypophyseal adrenotropic extracts. In 1937 Moon (5) described a method of assay of adrenotropic hormone which utilizes increase of adrenal weight in 21-day rats. More recently Moon (6) reported a modified method in which 4-day rats are utilized and much less material is required for assay.

The data of the present paper concern three aspects of this general problem. (*a*) It was found that under the influence of potent adrenotropic (pituitary) extracts the adrenal weight of 2-day chicks increases directly in proportion to the first power of the dosage used. This chick test requires less material than does the 21-day rat test, and this is important because of the low yield of adrenotropic material. (*b*) The preparation of active adrenotropic extracts from the pituitary is described. (*c*) The failure of non-pituitary substances including so-called alarm agents to stimulate the chick adrenal, and the similarity of results from normal and hypophysectomized chicks, suggest that the hypophysis of the chick has little power to make the adrenals enlarge; hypophysectomy thus seems unessential for critical work with chicks of this age.

MATERIAL AND METHODS

White Leghorn cockerels were used in all tests. They were obtained from two sources. Chicks from one source (C chicks), a local poultry dealer, came from a hatchery in Indiana. All of the data from this type of chick are presented in figure 2, and there only. All other chicks (M chicks) were obtained by us directly and throughout the year from a different source in Indiana (Martin's Ramsey Hatchery, Ramsey, Ind.). Chicks hatched presumably on Thursday, received on Saturday, were injected subcutaneously for 5 days beginning either on Saturday or Sunday. The 5-day period of dosage was selected because thyroid weights (also testis weights) were simultaneously followed according to the technic of Smelser (7). Thrice daily injections

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(morning and evening, on alternate sides; at midnight, in the neck) were used in most adrenotropic assays in order to conserve the supply of hormone.

The gonadotropic and thyrotropic potencies were estimated from the weights of testes and thyroids after once daily injections for 5 days into cockerels. The potency was read graphically from a standard curve established with preparation No. 630, 1 mg. of which was arbitrarily selected as containing 1 U of gonadotropic and 1 U of thyrotropic activity. In the case of both testis and thyroid 1 mg. of No. 630 approximately doubled the organ weight.

Prolactin unitage was obtained by the method of Riddle and Bates (8) on Carneau pigeons at 6 weeks after hatching.

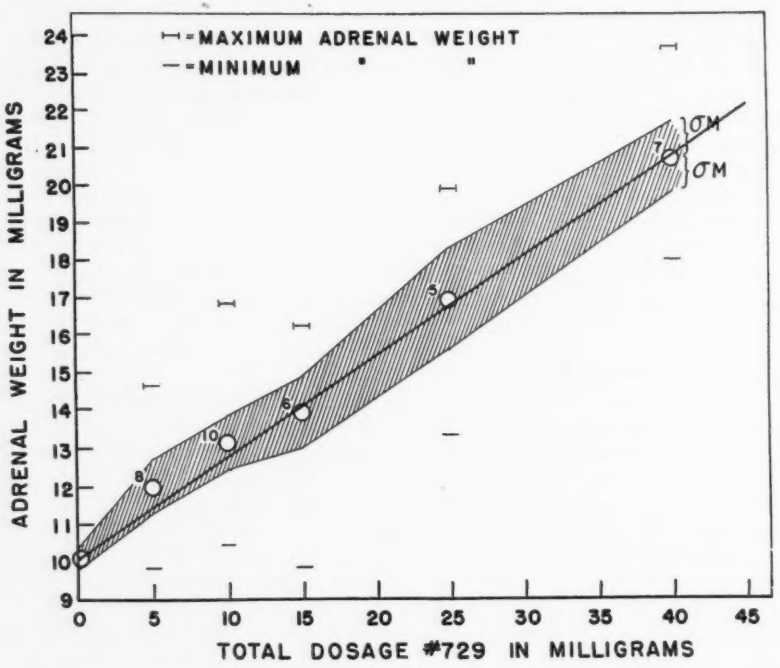

Fig. 1. Adrenal weight variation in 2-day-old White Leghorn cockerels. Preparation 729 injected thrice daily for 5 days.

Adrenal weights in 21-day rats are expressed as percentage increase in weight after 3 daily subcutaneous injections of 20 mg. each (a total of 60 mg.). This is essentially the original method of Moon (5). The control adrenal weight for 21-day rats in our colony is roughly 10 mg. in contrast to 16 to 17 mg. for the rats used by Moon.

Tryptophane was determined by the rapid method of Bates (9). Casein was used as a reference standard and was assumed to contain 2% tryptophane. If the tryptophane content of casein is 1.4%, as determined by the method of Folin and Ciocalteau (10), then our values should be reduced to seven-tenths that given in the tables published here.

Glucosamine was determined by a combination of the methods of Sørensen (11) and of Elson and Morgan (12).

RESULTS

Adrenotropic assay method. Experimental data testing the relationship between dosage and response (adrenal weight) are shown in figures 1 and 2, and in table 1. Adrenal weights were found to increase in proportion to the first power of the dosage whether dosage was once or thrice daily; a straight line therefore describes this relationship. The effectiveness of thrice daily injections was found to be almost exactly double that of any equal amount given at a single injection (fig. 2, table 1).

In 23 *M* chicks injected with 20 to 100 mg. of a muscle extract the adrenals weighed 9.9 ± 0.30 mg. with an extreme individual variation from 8.0 to 14.0 mg. Control adrenal weight for 19 *C* chicks was only 8.3 ± 0.28 mg. Control adrenal weights vary somewhat in different batches of *M* chicks. The highest average ob-

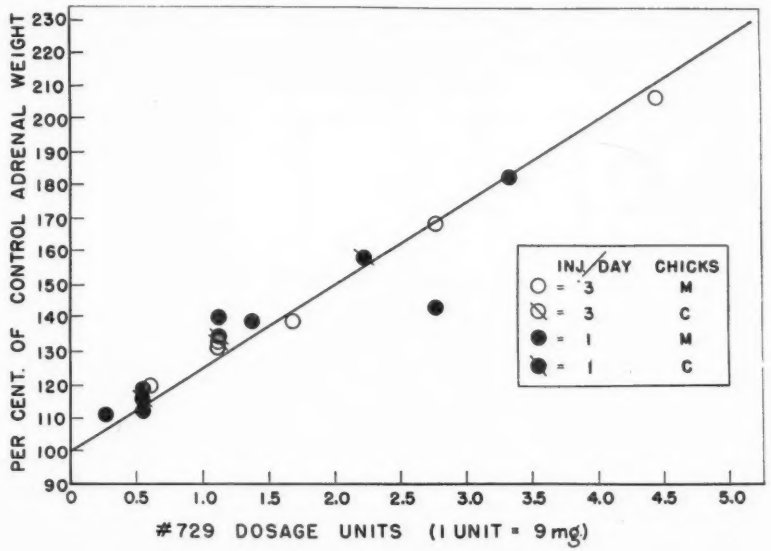

Fig. 2. Relation between adrenal weight response and dosage in two strains of 2-day-old White Leghorn cockerels. Injected once or thrice daily for 5 days (groups of 5–15).

tained for a group was 11.7 mg.; the lowest, 9.5. This variation necessitates control groups for each batch of chicks with conversion of adrenal weight into terms of percentage increase in adrenal weight. Within groups of 10 injected chicks the weight of the largest adrenal has exceeded twice the weight of the smallest adrenal only once—a case of high dosage where there was 100% increase in the average adrenal weight.

Our tests of the relation between dosage and response were made mostly with preparation 729 (see table 7 for further description). Figure 1 shows the maximum and minimum adrenal weights, mean adrenal weights, and the standard deviation of the means (shaded area), obtained at 5 different dosage levels of No. 729 injected thrice daily. The equation of the line which best fits the data (fig. 1) was determined by the method of least squares. This equation is: adrenal weight (mg.) $= 0.267 \times$ mg. of $729 + 10.1$. The variation in adrenal weights shown in figure 1 is typical, and the standard deviation is not shown in the tabulated data though it was calculated for

most groups. The standard deviation of the mean of the adrenal weights of groups of 10 chicks is usually less than ± 0.7 mg. at low levels of stimulation and rarely over ± 1.0 mg. at high levels where the adrenal weight is doubled. This means that a difference of 2 mg. in average weight of adrenals between 2 groups is significant if the adrenal weight increase is less than 50%.

Since 2 mg. is a 20% increase in weight of adrenals (for our chicks) we have selected a 25% increase in adrenal weight as a unit response. A unit of adrenotropic

TABLE 1. RELATIONSHIP BETWEEN DOSAGE AND ORGAN WEIGHTS OF ADRENAL, THYROID AND TESTIS IN SAME CHICKS WITH TWO DIFFERENT PITUITARY PREPARATIONS. AUGMENTATION OF EFFECTIVENESS BY THRICE DAILY INJECTIONS IS ALSO SHOWN

Injections		No. of chicks	Testes		Thyroids		Adrenals	
No. per day	Total dose, mg.		Weight, mg.	U[1]/mg.	Weight, mg.	U[1]/mg.	Weight	U[1]/mg. ×100
			Pork No. 638 pH 3–3.5 insol.					
3	10	10	8.3	0	3.3	0.10	12.1	8.5
3	20	10	9.0	0	7.7	0.30	12.7	5.4
3	40	9	9.8	0	7.7	0.15	14.7	4.8
3	60	10	11.8	0	9.8	0.18	17.0	4.7
3	100	7	19.0	0.006	10.1	0.12	27.2	6.7
3	40+2 mg. $ZnSO_4$	10	11.1	0	6.1	0.10	16.3	6.3
							Ave.	6.1
			No. 669 Whole beef extract					
3	5	8	20.9	0.180	12.7	6.00	11.7	13.0
3	10	8	27.6	0.200	12.6	3.00	12.0	8.0
3	20	5	22.7	0.060	12.7	1.50	14.3	8.7
3	40	7	33.6	0.125	14.7	1.25	14.3	4.3
3	40	8	36.8	0.200	16.3	1.75	15.6	5.6
3	80	1	44.3	0.250	10.6	0.20	22.6	6.3
			Ave.	0.150			Ave.	7.6
			No. 669 Whole beef extract					
1	10	10	16.4	0.050	4.9	0.2	11.6	6.3
1	10	6	12.7	0.025	6.4	0.4	10.8	3.3
1	20	7	20.1	0.035	8.1	0.4	11.8	3.7
1	40	8	21.0	0.022	13.6	0.9	11.7	1.6
1	40	10	34.4	0.150	20.8	5.0	13.8	3.8
			Ave.	0.056			Ave.	3.5

[1] Units uncorrected for thrice daily injection

activity is contained in the total amount of material which produces a 25% increase in adrenal weight when injected subcutaneously thrice daily at approximately 8-hour intervals for 5 days into White Leghorn cockerels. Two units would produce a 50% increase under the same conditions but only a 25% increase if injections were made once daily. For most accurate assay we recommend the use of sufficient extract to produce a 50% increase in adrenal weight.

All data obtained with injections of 729 for 5 days in both M and C chicks are plotted in figure 2. Because of the discrepancy in unstimulated adrenal weight between M and C chicks the weights obtained are expressed as percentage of unstimulated adrenal weight. The dosages of the groups injected once daily have been divided by 2 to make them conform with the thrice daily injected groups—this

procedure being based on our finding that there is a doubling of effectiveness upon dividing the dosage in 3 parts daily. With the exception of one low result the agreement with the theoretical solid line (% increase in adrenal weight ÷ 25 = units) is excellent.

The adrenal weights obtained with increasing dosage of 2 other preparations are shown in table 1. Thrice daily injections of No. 669, an unfractionated extract, are seen to be 2.2 times as effective for increasing adrenal weight as once daily injections. Average weights of testes and thyroids from the same chicks, together with the units per mg. of gonadotropin and thyrotropin calculated therefrom, are recorded to show the further usefulness of the chick as a test animal. The graphs constructed for our determination of the unitage of the 2 last-named hormones are based on once daily injections; augmentation with thrice daily injections is seen in table 1 to be nearly 3 times for gonadotropin, and much greater (perhaps 5 to 10 times) for thyrotropin in tests thus made with 669. Thyroid weights over 12 mg. are in the region of maximum

TABLE 2. EFFECT OF NON-PITUITARY HORMONES ON ADRENAL AND THYMUS WEIGHT IN CHICKS. ALL INJECTED FOR 5 DAYS

Preparation	No. injections per day	Total dose	No. of chicks	Weight	
				Adrenals	Thymus
				mg.	*mg.*
Thyroxin	1	250γ	4	11.6	—
	1	500γ	2	10.7	61
Insulin	2	1 U	7	10.3	—
	2	2 U	5	11.6	46
Gamone	3	15 mg.	3	10.7	31
Estrone (in oil)	1	1.7 mg.	5	9.7	41
Dihydroestrone	1 pellet		5	11.0	—
Testosterone propionate	1 pellet		5	11.2	92
Desoxycorticosterone acetate	1 pellet		4	10.7	51
Desoxycorticosterone acetate (in oil)	1	5 mg.	5	9.0	45

response (for *M* chicks); consequently all thyroid weights on thrice daily injections of 669 come under this category and the unitages calculated therefrom are not significant except in their indication of a great augmentation of effectiveness. It is readily seen that when the unitages obtained with 638 are corrected to once (from thrice) daily injection this preparation contains only 0.002 times as much gonadotropin and only 0.02 to 0.04 times as much thyrotropin as our standard, 630. When a preparation contains as little gonadotropin and thyrotropin as 638 satisfactory results are obtained at the high level of dosage required for adrenal assays; otherwise reassay at a total dosage level of only 1 to 5 mg. in once-daily injections is necessary for a more accurate assay of these particular hormones.

Effect of non-pituitary substances on the chick adrenal. Many non-pituitary substances have been found to enlarge the adrenal cortex in intact animals but usually not in hypophysectomized animals. This is commonly interpreted as indicating that these substances cause release from the hypophysis of adrenotropic hormone which in turn acts on the adrenal cortex. For this reason Selye (13) has emphasized the necessity of using hypophysectomized animals in tests for adrenotropin. Associated with adrenal hypertrophy in the syndrome which Selye (14) has called the "alarm reaction" is thymus atrophy. Consequently we have included thymus weights (from one side of neck only) as confirmatory evidence (table 2, 3) for the slight effect of non-pituitary substances on adrenal weight of young chicks. Control thymus weights vary with different batches of chicks from 40 to 80 mg. so that thymus weights below

40 mg. indicate definite atrophy. The thymus weight is usually less than 40 mg. when dosages of adrenotropic preparations are sufficient to increase the adrenal weight by 25%.

The high dosages of thyroxin, insulin, gamone, formaldehyde, copper and zinc sulfates, and (probably) ammonia used in our tests were nearly the maximum which would permit survival for 5 days. In these cases doubling of the dosage would have been lethal. In spite of such near-lethal dosage there is only one case in tables 2 and 3 where the increase in adrenal weight (to 12 mg.) was more than 20% and that case (lowest dose of formaldehyde) was not confirmed definitely by the other subsequent tests even at higher dose levels. This one high value was associated with a uniquely large increase in body weight which partly accounts for the magnitude of the final weights of both adrenal and thymus; but in the other tests with formaldehyde there is a suggestion of stimulation in adrenal and atrophy of thymus. With

TABLE 3. EFFECT OF NON-PITUITARY SUBSTANCES ON ADRENAL AND THYMUS WEIGHT IN CHICKS. ALL INJECTED FOR 5 DAYS

Preparation	No. injections per day	Total dose	No. of chicks	Weight	
				Adrenals	Thymus
				mg.	mg.
	1	1 cc. of 2%	4	13.9	96
	1	1 cc. of 2%	10	9.7	47
Formaldehyde	3	1 cc. of 2%	5	10.6	40
	3	1 cc. of 4%	5	11.5	28
	3	1.5 cc. of 4%	3	11.9	38
$CuSO_4$	1	1 mg.	4	11.0	68
$ZnSO_4$	1	5 mg.	2	10.0	—
	1	2 mg.	5	11.3	60
KCl	1	20 mg.	5	8.7	58
Casein	1	100 mg.	5	8.9	53
NH_4OH	1	1 cc. of 1%	5	10.3	25

none of these substances is the degree of stimulation obtained such as would appreciably affect results of assay of pituitary extracts where a 50% increase in adrenal weight (to 15 mg.) is utilized for assay purposes.

Since our results indicate ineffectiveness of the above-mentioned non-pituitary substances in young chicks only brief reference will be made here to literature on this subject. Hypertrophy of the adrenal cortex has been frequently reported following the administration of desiccated thyroid or thyroxin since Hoskins (15) first described this effect. A recent paper by Miller and Riddle (16) reviews the literature and describes the action of insulin, gamone, thyroxin and estrone on the adrenals of rats and pigeons. The effect of androgens on adrenals is reviewed by Koch (17). Wells and Kendall (18) have recently reported that desoxycorticosterone acetate does not cause atrophy of either adrenal cortex or thymus in rats. Formaldehyde was here examined because it is one of the "alarm" reagents of Selye, and ammonia was tested because of the adrenal hypertrophy reported by Fazekas (2) from its oral administration in rabbits. Inorganic salts and casein were injected as control substances for other tests.

Effect of thyroidectomy and hypophysectomy. Several investigators (19, 20, 21) have concluded that thyrotropic hormone causes adrenal enlargement through increased production of thyroid hormone, and state that thyroidectomy blocks this action. Others (22–25) report that the presence of the thyroid is not necessary for adrenal hypertrophy with adrenotropin. Since non-specific adrenal stimulating substances,

as well as thyrotropin, are believed by some to act through stimulation of the pituitary it was necessary to learn whether or not the adrenal enlargement observed in the intact birds occurs also in hypophysectomized chicks.

Our results with thyroidectomized and hypophysectomized chicks are given in table 4. Thyroidectomy altered the adrenal weight response appreciably neither when a prolactin preparation (734) nor when highly potent thyrotropic preparations (642, 730, 782; see table 6, 7) were used. Much difficulty was experienced in keeping hypophysectomized chicks alive; half of the birds usually died by the third day and most of the others by the fifth day. Potent pituitary preparations stimulated the adrenals of these hypophysectomized chicks nearly as much as those of normal chicks though body weights were reduced by as much as 20 to 25%.

TABLE 4. EFFECT OF THYROIDECTOMY AND HYPOPHYSECTOMY ON ADRENAL RESPONSE IN CHICKS. ALL OPERATED 5 DAYS BEFORE AUTOPSY AND INJECTED THRICE DAILY

No. of preparation	Description	Total dose, mg.	No. of chicks	Adrenal weight		Change
				Mg. expected	Mg. found	
		Thyroidectomized				
	Uninjected	—	4	10.0	11.0	+10%
	Uninjected	—	4	10.0	11.7	+17%
734	Prolactin	60	5	14.5	13.7	− 6%
642	Cu-insoluble	40	4	13.6	15.2	+12%
730	Cu-insoluble	20	2	16.2	15.9	− 2%
782	ppt. on dialysis	20	6	18.0	20.6	+12%
		Hypophysectomized				
	Uninjected	—	4	10.0	9.9	− 1%
669	Whole gland extr.	40	2	15.6	16.2	+ 4%
763	pH 3.5 insoluble	40	5	14.8	11.8	−20%
782	ppt. on dialysis	20	4	18.8	16.7	−11%

Our results with chicks essentially parallel those obtained on 21-day rats by Moon (5, 25) in that (*a*) thyroid hormone has no appreciable effect on adrenal weight, (*b*) thyroidectomy does not alter the magnitude of the adrenal stimulation with adrenotropic extracts, and (*c*) hypophysectomy does not prevent the action of the extracts.

Another point of agreement of data from chicks and rats requires emphasis. Most responses to hormones show a proportionality to the logarithm of dosage. The simple direct relationship of adrenal weight increase to the first power of the dosage found here in chicks, and also in rats as shown by our inspection of data published by Moon (5, 25, 26), is unusual and probably significant.

Preparation of adrenotropic fractions. Over a period of 3 years we have used the 21-day rat to test our various pituitary preparations for adrenotropic activity. When potent preparations appeared they were further fractionated by various methods in attempts to separate prolactin, gonadotropin and thyrotropin from adrenotropin. Results were discouraging because of the apparent absence of adrenotropic potency from most of the larger fractions thus secured and because no fraction more potent than the starting material was obtained. The only significant finding from all this work was that when pH 3.5 soluble or isosoluble fractions were dialyzed precipitates were formed which uniformly contained more adrenotropic activity per mg. than other fractions. Such fractions, however, always stimulated crop-sacs, testes and thyroids in addition to adrenals (table 7).

TABLE 5. SOME PHYSIOLOGICAL AND CHEMICAL PROPERTIES OF AQUEOUS ISOELECTRICALLY INSOLUBLE FRACTIONS FROM PITUITARIES. (ESSENTIALLY FREE OF GONADO- AND THYROTROPIC ACTIVITY)

No. preparation	Description	Pigeon		Chicks		Rat	Ash	Tryptophane	Glucosamine
		Prolactin	Gonadotropin	Thyrotropin	Adrenotropin	Adrenal increase			
		u/mg.	u/mg.	u/mg.	u/mg. ×100	%	%	%	%
706	Prolactin	7.0	<0.005	<0.005	2.3	0	1.2	2.5	0.17
734	Prolactin	4.4	<0.005	<0.005	3.0	10	1.0	2.5	0.24
L.537	pH 6.5 ppt.	2.0	<0.005	<0.005	7.3	50	1.2	2.0	0.31
L.538	pH 6.5 sol.	1.5	<0.005	<0.005	4.6	20	2.6	1.6	—
L.572	pH 6.5 sol.	0.5	<0.005	0.025	13.8	38	2.6	2.5	0.44
SL.576	pH 6.5 ppt.	1.0	<0.005	<0.005	3.6	70	2.8	2.8	—
SL.577	pH 6.5 sol.	1.0	<0.005	<0.005	12.0	20	8.4	2.1	0.30

L= prepared by Lyons' method; S= prepared from sheep glands; all others from beef glands.

Tables 5, 6 and 7 summarize our data on the hormonal content and chemistry of some of our more recent preparations; the 3 tabulations classify preparations according to their solubility. The results of assays of adrenotropin as obtained on both chicks and rats are given. The quantitative agreement in the 2 animal types is poor for preparations 572, 577, 764, 781 and 782; in a general way, however, the 2 methods give like results. Table 5 shows that the isoelectrically insoluble fractions of prolactin prepared by our method (27), and the pH 6.5 insoluble and soluble fractions prepared by the method of Lyons (28), are essentially devoid of gonadotropin and thyrotropin and contain relatively little glucosamine and much tryptophane. All of these properties are characteristic of prolactin preparations. The adrenotropic content of prolactin is low in the 2 prolactin preparations (706, 734) and it could be further reduced. It is our experience that much of the adrenotropic activity passes into the pH 6.5 soluble fraction.

Anterior pituitary fractions which are most soluble in water (table 6) contain much glucosamine, and only one-third as much tryptophane, as the isoelectrically insoluble fractions. They also contain no prolactin but are rich in gonadotropic, thyrotropic and, sometimes, in adrenotropic activity. The so-called copper-soluble and -insoluble fractions in table 6 were fractionated by the method of Bates and Riddle (29). This copper treatment in one step quite completely removes prolactin

TABLE 6. SOME PHYSIOLOGICAL AND CHEMICAL PROPERTIES OF SO-CALLED AQUEOUS ISOELECTRICALLY SOLUBLE FRACTIONS FROM PITUITARIES. (ESSENTIALLY PROLACTIN FREE)

No. of preparation	Description	Pigeon	Chicks				Rat	Ash	Tryptophane	Glucosamine
		Prolactin	Gonadotropin	Thyrotropin	G/T	Adrenotropin	Adrenal incr.			
		u/mg.	u/mg.	u/mg.		u/mg. ×100	%	%	%	%
P534	Cu-insoluble	<0.000	0.250	0.30	0.90	5.4	57	6.0	0.40	2.2
630	Cu-soluble	<0.000	1.000	1.00	1.00	5.3	0	4.0	0.77	8.1
P642	Cu-insoluble	0.000	0.600	3.00	0.20	4.0	40	2.4	1.00	{2.0 / 2.5}
P643	Cu-soluble	0.000	5.000	0.50	10.00	3.0	10	3.8	0.80	9.2
730	Cu-soluble	0.002	0.800	1.20	0.67	12.4	60	0.4	1.10	8.5
731	Cu-insoluble	0.030	0.500	2.80	0.18	13.7		1.3	1.10	5.5
P747	Sol. on dial.	<0.100	5.000	5.00	1.00	<2.0	25	2.0	1.05	6.2
P749	Sol. on dial.	<0.050	1.500	3.50	0.43	<2.0	15	<1.0	1.46	7.0
762	pH 3.5 sol.	<0.100	1.000	5.00	0.20	13.7	66	42.0	0.87	4.2
764	pH 3.5 sol.	0.100	<0.005	<0.01	?	14.0	47	55.0	1.17	3.3
764C	Sol. on dial.	<0.050	0.200	0.02	10.00	48.0	—	<1.0	1.22	3.7
781	Sol. on dial.	<0.030	0.130	0.05	2.60	19.0	40	<1.0	1.18	4.2
783	Sol. on dial.	<0.030	1.800	1.50	1.20	12.0	59	<1.0	0.70	5.4

P= made from pork glands.

TABLE 7. SOME PHYSIOLOGICAL AND CHEMICAL PROPERTIES OF FRACTIONS OBTAINED UPON DIALYSIS OF AQUEOUS ISOELECTRICALLY SOLUBLE FRACTIONS

No. of preparation	Description	Pigeon	Chicks				Rat	Ash	Trypto-phane	Glucos-amine
		Prolactin	Gonado-tropin	Thyro-tropin	G/T	Adreno-tropin	Adrenal increase			
		u/mg.	u/mg.	u/mg.		u/mg. ×100	%	%	%	%
629	ppt. on dial.	0.40	0.120	0.08	0.15		55	7.0	1.40	
P641	ppt. on dial.	0.25	1.000	0.70	1.40		79	6.6	1.00	1.4
S645	ppt. on dial.	1.00	0.040	0.80	0.07		107	4.4	1.30	1.9
660	ppt. on dial.	0.50	*	*			135	3.8	2.00	0.9
661	ppt. on dial.	2.00	*	*			134	2.0	2.50	1.1
729	ppt. on dial.	0.25	0.200	3.00	0.07	11.1	119	1.2	1.76	4.0
732	(Cu-insol.) dial.	0.20	0.400	4.00	0.10	12.8	76	3.2	1.10	4.4
P750	ppt. on dial.	0.50	1.500	6.20	0.24	7.5	65	1.0	1.46	4.6
764B	ppt. on dial.	0.15	0.005	0.01	—	30.0	—	1.0	1.94	1.9
780	ppt. on dial.	0.20	0.010	0.02	0.50	23.0	95	6.6	1.91	1.8
782	ppt. on dial.	0.03	0.550	2.00	0.27	14.8	39	4.6	1.10	3.1

P=from pork glands; S=from sheep glands; all others from beef glands.
* Potent but not assayed on chicks.

but apparently not adrenotropin from such fractions. Preparations 630, 642 and 643 were found to contain only slight amounts of adrenotropin compared with 629 and 641 the corresponding precipitates from the dialysis which is preparatory to the copper treatment (table 7). The apparent separation effected by dialysis in the above cases has not since been confirmed in the 729–730–731–732 series, the 764 series, the 780–781 series or the 782–783 series of preparations.

The properties of the precipitates formed on dialysis of various fractions are shown in table 7. These fractions are intermediate to the 2 groups just described in most of their properties, and they usually have high adrenotropic activity.

The method of preparation of fractions shown in table 8 was mainly that of Bates and Riddle (27). The starting material was 10 lb. of beef anterior lobes that had been stored under acetone. The extraction at PH 9.5 with 65% ethanol was repeated once and the extracted material then divided into what was approximately 2 equal parts by removal of the precipitate obtained upon acidification to PH 6 (No. 766) before 2 volumes of ethanol were added in order to precipitate the prolactin (No. 767). Fraction 767 contained most of the prolactin and adrenotropin with little of the other 2 hormones, while 766 was a mixture of all the 4 hormones tested. There was also

TABLE 8. DISTRIBUTION OF ADRENOTROPIC ACTIVITY ON FRACTIONATION

No.	Fraction Description	Wt.	Ash	Trypto-phane[1]	Glucos-amine[1]	Prolactin		Gonadotropin		Thyrotropin		Adrenotropin	
							Total		Total		Total		Total
		gm.	%	%	%	u/mg.	u	u/mg.	u	u/mg.	u	u/mg. ×100	u
766	PH 6 65% ethanol-insoluble	27.0	11	1.25	1.6	1.00	27,000	0.200	5,400	0.60	10,000	4.5	1,200
775	PH 3.5 insol. from 766	20.0	5	1.30	1.2	1.00	20,000	0.006	120	0.09	1,800	<2.0	<400
776	PH 3.5 sol. from 766	7.0	44	0.53	3.7	0.04	280	1.700	11,900	0.20	1,400	11.0	770
782	ppt. on dialysis of 776	1.3	5	1.10	3.1	<0.03	—	0.550	715	2.00	2,600	14.8	192
783	sol. on dial. of 776	2.1	<1	0.70	5.4	<0.03	—	1.800	3,800	1.50	3,150	12.0	252
767	PH 6 { 65% ethanol soluble / 85% ethanol ppt.	35.0	5	2.1	0.9	10	350,000	0.010	350	0.02	700	7.0	2,450
772	PH 3.5 insol. from 767	26.0	9	2.2	0.5	11	286,000	0.003	—	<0.01	—	2.0	520
771	PH 3.5 sol. from 767	7.0	36	1.4	3.8	0.05	350	0.016	112	0.04	280	21.5	1,505
780	ppt. on dialysis of 771	1.8	6	1.9	1.8	0.2	360	0.010	18	0.02	36	23.0	414
781	sol. on dialysis of 771	2.3	<1	1.2	4.2	<0.03	—	0.130	299	0.05	115	19.0	437

[1] Corrected for ash content.

considerable difference in the tryptophane and glucosamine content of the 2 fractions.

These 2 fractions, 766 and 767, were then separately extracted twice in water at pH 3.5 according to our usual procedure, using H_2SO_4 (and adding $NaSO_4$ to 0.5% on reprecipitation), and the pH 3.5 soluble fractions (which do not form a precipitate upon addition of NaOH) were precipitated by addition of 4 volumes of acetone and the precipitate dried. The pH 3.5 insoluble fractions were also dried with acetone. These fractions and their properties are shown in table 8. The significant and consistent fractionation at pH 3.5 is shown by the hormonal content of the derived fractions and also by their distinctly different glucosamine and tryptophane content. Chance, Rowlands and Young (30) have recently confirmed the completeness of this separation at pH 3.5. Most of the adrenotropin appeared in the pH 3.5 soluble fractions along with the gonadotropin and thyrotropin. These two pH 3.5 soluble fractions were dialyzed at pH 4–6 to remove the contaminating salt and thereby (in dialysis) obtain further fractionation. The precipitates which formed upon dialysis were dried with acetone and the supernatant solutions were treated with 4 volumes of acetone and their precipitates dried.

In the above manner 4 final adrenotropic fractions (780, 781, 782 and 783) were obtained whose other properties were quite distinct. No. 780 had the highest content of tryptophane, prolactin and adrenotropin and lowest content of glucosamine, gonadotropin and thyrotropin; 783 had the highest content of glucosamine, gonadotropin and thyrotropin and the lowest content of tryptophane and adrenotropin. However, the adrenotropin content of 780 is only twice that of 783. It is apparent from a consideration of the results shown for these fractions (table 8) that adrenotropic activity is not necessarily associated with prolactin, gonadotropin, thyrotropin, or with a fraction insoluble at pH 6.5.

The yields of the various hormones as indicated in the columns for "total units" in table 8 show consistent results—with little loss—for prolactin; consistent results—with about 20 to 40% loss of adrenotropin at each fractionation; and somewhat variable results for gonadotropin and thyrotropin. We believe that the indicated yields reflect the accuracy and specificity of the various tests that we have used; the test used for gonadotropin being probably the least accurate and reliable.

A 2-pound batch of beef glands was processed earlier in a similar manner. Fractions 762 and 764 (table 6) from this batch correspond to 776 and 771, respectively; 764*B* and 764*C* correspond to 780 and 781, respectively. The yields of these fractions, and the results of hormonal assays and of the chemical studies made upon them, were in all respects similar to those obtained from the larger batch of glands described above (table 8). As measured on chicks the highest adrenotropic potency till now obtained by us was present in 764*B* and 764*C*; assays on rats could not be made with these preparations because such assays alone would have required the entire 300 mg. obtained in each fraction.

Preparations 764*B* and 780 are the purest adrenotropic preparations made and tested by us. They contain less than one part in one hundred of each of the 3 other pituitary hormones measured. Microscopic study of chick adrenals treated with preparations 764*B* and 780 (and still other adrenotropic preparations) shows both hypertrophy and hyperplasia of the cortical cells. A rather striking increase in the vascularity of adrenals showing higher responses is also found.

General statement on adrenotropin. One could wrongly conclude from the Lyons' (28) method of preparing adrenotropin that it is a typical aqueous isoelectrically insoluble protein which is fairly soluble in aqueous acetone or ethanol like insulin, prolactin or casein. Collip's (31, 32) best adrenotropic preparations were soluble in

70% ethanol and were precipitated isoelectrically from water at pH 6 only after long standing. It is thus somewhat anomalous that the chief known contaminant in Collip's preparation was thyrotropin, while in Lyons' preparations the chief contaminant was prolactin. Jensen and Grattan (33) have recently specified precipitation of adrenotropin at pH 6.5 with 0.2 saturated ammonium sulfate as a means of removing thyrotropin. This may indicate solubility of adrenotropin at pH 6.5 in the absence of the sulfate. Aqueous acetic acid extracts (Loeb extract), which contain little prolactin, have been used for adrenotropic tests (21, 34), and Perla (34) has reported adrenotropic action in the pH 6.5 soluble fraction from such an extract.

The results of others and our own experience recorded in this paper seem to us to indicate that the adrenotropic principle is like prolactin in that it has an appreciable solubility in 60 to 70% ethanol or acetone, even in the isoelectric region, which differentiates it from gonadotropin and thyrotropin. It is unlike prolactin and like gonadotropin and thyrotropin in its high solubility at pH 3.5 in the presence of sulfuric acid. The high adrenotropic potency of our fractions that were soluble on dialysis seems contrary to the work of others. The pH 3.5 procedure applied to a Lyons pH 6.5 adrenotropic fraction should give further valuable information.

The low yield of highly potent adrenotropic fractions and the large amounts of this material required for assay have greatly retarded work on this important subject.

It will be noted that in our assays we have made no attempt to sub-divide gonadotropic activity into FSH and LH. We have presented no data which would exclude LH as causative agent of this response of the adrenal of the chicks and rats used for assay, and some of our best adrenotropic preparations were found to cause marked stimulation of the seminal vesicles of 21-day rats. Since potent preparations inhibit growth, in our own tests and in those of Moon (35), a "growth" hormone is obviously not involved. Opinion on the question of the rightful name of the "adrenotropic" principle is reserved.

SUMMARY

A method for assaying adrenotropic preparations on 2-day chicks is described. Chicks are injected subcutaneously daily for 5 days. The total amount of a pituitary preparation required to increase adrenal weight by 25% when injections are thrice daily, or one-half the amount required for a 25% increase when injections are only once daily, was selected as the unit of adrenotropic or corticotropic activity. In chicks, as in rats, adrenal weight increases in proportion to the first power of the dosage. Potent preparations induce both hypertrophy and hyperplasia of cortical cells.

Non-pituitary substances, either hormonal or otherwise, when administered by injection at nearly lethal dosage levels cause little or no enlargement of the adrenals of these chicks. The substances tested were thyroxin, insulin, estrone, dihydroestrone, testosterone, desoxycorticosterone, formaldehyde, ammonia, casein, zinc and copper sulfates, potassium chloride.

Thyroidectomy did not alter the degree of the adrenal response even when pituitary fractions with high thyrotropic content were used. Hypophysectomy did not prevent adrenal enlargement although it tended to decrease the degree of the response.

A method of preparing highly potent adrenotropic fractions essentially free from prolactin, thyrotropin and gonadotropin is presented. The method is based on solubility in 60% ethanol and at pH 3.5 in water with subsequent division of the pH 3.5 extract into soluble and insoluble fractions by dialysis.

The possible identity of this principle with still other pituitary principles is considered.

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INTERSTITIAL CELL STIMULATING HORMONE
I. BIOLOGICAL PROPERTIES[1]

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THE SECRETION by the pituitary of two hormones with specific effects on the gonads has been recognized for some time. While the properties of one of these, the substance stimulating ovarian follicular development (FSH), have been sufficiently characterized, no such general agreement has been reached regarding the other component.

Fevold has described the isolation of a luteinizing hormone, "LH" (1). This substance either alone or in combination with FSH (the latter is the more recent opinion of the author (2)) increases the weights of testes and accessories in immature normal and hypophysectomized male rats and may be measured by its effect on the seminal vesicles; another method of assay suggested by this author is based on his finding that LH in combination with FSH very strikingly increases the ovarian weights over those produced by FSH alone, an effect called "augmentation." For both tests, a doubling of weights (100% increase) has been defined as a unit, greater effects being produced with higher doses. It is noteworthy that luteinization is no longer the criterion for a quantitative assay of the potency of 'LH' solutions. Our observations indicate clearly that the seminal vesicle and ovarian weight-augmenting potency of solutions does not always parallel their luteinizing ability and the term 'LH' will be used throughout this study to designate the hormone responsible for weight augmenting effects on seminal vesicles and ovaries without reference to effectiveness in the formation of corpora lutea.

It is well known that some investigators have attempted to explain the effects of the second gonadotropic substance as due merely to non-specific augmentation of the FSH effect, similar to that which can be obtained on injecting this hormone mixed with the salts of heavy metals or with certain inert proteins (1, 4) but this contention is readily negated by the injection of the two gonadotropic factors at different sites.

This laboratory has announced the identification in pituitary extracts of a gonadotropin which may be measured by its ability to repair the interstitial tissue of the ovary of hypophysectomized rats, ICSH (3). The hormone resembles Fevold's LH in that it increases, though to a lesser degree, the weight of the accessories in males and augments the effect of FSH on the ovary in the female.

ICSH has now been obtained in pure form and while the method of preparation of the hormone and its physico-chemical properties will be given in the following paper, its biological effects will be reported here.

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TABLE 1. AUGMENTATION OF FSH BY ICSH (NORMAL IMMATURE RATS)
(3 rats per group unless otherwise stated)

Total dose		Days of injection (subcut.)	Ovaries		Uterus
FSH[1]	ICSH[1]		Weight	Description	Description
R.U. (*IF 108F*) (1 U=0.02 mg.)	R.U. (*L51E II*)		*mg.*		
7·5	—	3	43	m to lF	l
7·5	1	3	49	lF	l, est. (1 rat)
7·5	5	3	52	lF	l, est. (1 rat)
7·5	10	3	60	lF, CL (1 rat)	m to l
7·5	50	3	53	CL (3 rats)	m
7·5	100	3	48	CL (3 rats)	m
(*SV13B*) (1U=0.025 mg.)	(*L 61 F*)				
20	—	3	42	lF, CL (2 rats)	l, est. (2 rats)
20	5	3	62	lF, CL (3 rats)	l
20	20	3	53	lF, CL (2 rats)	m to l
20	100	3	43	lF, CL (3 rats)	m
10	—	3	44	lF	l, est. (2 rats)
10	5	3	55	lF	l, est. (2 rats)
10	20	3	53	CL (3 rats)	m to l
10	100	3	47	m to lF, CL (3 rats)	m to l
4	—	3	29	smF	s to m
4	5	3	33	lF	l, est. (2 rats)
4	20	3	55	lF, CL (2 rats)	l, est. (1 rat)
4	100	3	45	m to lF, CL (3 rats)	m
2	—	3	22	smF	s to m
2	5	3	29	m to lF	ml, est. (1 rat)
2	20	3	32	m to lF	m
2	100	3	32	m to lF, CL (3 rats)	m
(*IIF 36 A*) (1 U=0.01 mg.)	(*L 65 B*)				
5	—	5	45	mF	m to l
5	1	5	48	m to lF	est.
5	5	5	72	m to lF, CL (2 rats)	l
5	10	5	71	m to lF, CL (1 rat)	m to l
(*IIF 43 D*) (1 U=0.02 mg.)	(*L 79 C*)				
3·75	—	5	41[2]	lF	m to l
3·75	1.0	5	42[3]	lF, CL (1 rat)	l, est. (3 rats)
3·75	2.5	5	53[3]	m to lF, CL (2 rats)	l, est. (2 rats)
3·75	7·5	5	98[3]	lF, CL (3 rats)	l
3·75	10.0	5	41[3]	m to lF, CL (1 rat)	l, est. (3 rats)

[1] One rat unit of FSH is the minimal total dose which on subcutaneous injection once daily for 3 days causes resumption of ovarian follicular development in hypophysectomized rats (26–28 days of age at operation, 6–8 days postop. at onset of injection). One rat unit of ICSH is the minimal total dose which on intraperitoneal injection for the same time and in the same type of rats causes repair of the ovarian interstitial tissue. Unless otherwise stated, pure ICSH preparations were employed throughout, 0.01 mg. of which corresponds to 1 U.

[2] 4 rats per group.

[3] 5 rats per group.

Key to Abbreviations

F=follicles
CL=corpora lutea
est.=estrous uterus
do=distended oviduct
db=distended bursa
incr.=increase in
sev.=several
s=small
m=medium
l=large
I.T.=interstitial tissue
−=deficient
+=repaired
2+=hypertrophied

TABLE 2. AUGMENTATION OF FSH BY ICSH (HYPOPHYSECTOMIZED IMMATURE RATS)

Total dose		No. of rats	Days of injection (subcut.)	Ovaries		Uterus
FSH[1]	ICSH[1]			Weight	Description	Description
R.U. (*IIF 43 D*) (1 U=0.02 mg.)	R.U. *L79C*			*mg.*		
2.5	—	3	5	28	m to lF	l
2.5	2	3	5	36	m to lF	est.
2.5	4	3	5	62	m to lF, CL	est.
2.5	10	3	5	33	m to lF	est.
(*IIF 37 D*) (1 U=0.02 mg.)	(*L 61 F*) —	2	3	31	m to lF	m to l
5	1	2	3	44	l	ml
5	5	2	3	28	m to lF	est.
5	25	2	3	64	lF, CL (1 rat)	est.
5	100	2	3	67	mF, CL (2 rats)	est., m
	(*L 79 C*)					
5	1	5	5	48	m to lF, smCL (2 rats)	est.
5	5	5	5	46	m to lF, CL (2 rats)	est.
5	25	3	5	71	lF, CL (2 rats)	l, est. (1 rat)
5	50	5	5	50	m to lF, CL (2 rats)	m, est. (2 rats)
	(*L 61 F*)					
5	1	5	5	33	m to lF	m, est. (3 rats)
5	5	5	5	38	m to lF, CL (2 rats)	l, est. (4 rats)
5	25	3	5	78	lF, CL (2 rats)	l, est. (1;rat)
5	50	5	5	50	m to lF, CL (3 rats)	l, est. (4 rats)
(*IIIF 72 BD*)[2] (1 U=0.006 mg.)	(*L141DTP*)					
2.5	—	4	5	26	m to lF	s tom,est. (1rat)
2.5	0.5	2	5	35	m to lF	est.
2.5	1.0	2	5	45	lF	est.
2.5	2.5	2	5	43	m to lF	est.
2.5	5.0	2	5	49	lF	est.
2.5	10.0	2	5	67	mlF, CL (1 rat)	est.
2.5	25.0	2	5	61	s to mF, CL	l, est.
(*IVF 20 B*)[2] (1 U=0.004 mg.)	(*L141DTP*)					
2.5	—	2	4	19	mlF	s
2.5	0.25	3	4	19	mF	s
2.5	0.50	3	4	18	m to mlF	est. (1 rat)
2.5	1.00	3	4	15	m to lF	est. (1 rat)
2.5	2.50	3	4	35	lF	est.
2.5	5.00	3	4	27	lF	m, est. (2 rats)
2.5	10.00	3	4	36	lF, CL (1 rat)	est.
2.5	—	7	5	20	s, m, lF (2 rats)	est. (2 rats)
2.5	0.25	3	5	20	s to m	est. (1 rat)
2.5	0.50	5	5	28	lF	est.
2.5	1.00	5	5	37	m to lF	est.
2.5	2.50	4	5	32	m to lF	est.
2.5	5.00	2	5	21	sm, lF	est.
2.5	10.00	2	5	35	lF	est.
2.5	25.00	2	5	19	sF	smF

[1] For definition of units, etc., see footnote on table 1.

[2] Injection of FSH and ICSH at different sites of the body (in all other experiments the hormone solutions were mixed in vitro before injection). For method of preparation of *IIIF 72 BD* see (7).

TABLE 3. EFFECT OF PITUITARY FRACTIONS ON SEMINAL VESICLE WEIGHTS OF RATS

Fraction	Total dose protein	Equivalent of fresh gland	Seminal vesicle weights[1]	Increase
	mg.	mg.	mg.	%
Saline extract of glands	100	1000	20.0	63
	50	500	19.7	60
	20	200	13.4	9
	22[2]	220	14.5 (H)	90
Alkaline extract (NaOH, pH 8–9) of glands	40	200	17.0	38
2% Pyridine extract of glands	10.0	400	15.5	26
	2.0	80	14.0	14
	0.5	20	12.0	—
Acetone dried glands, alkaline extract (pH 8–9)[2]	175.0	2500	40.2	226
	—	250	14.3 (H)	88
	—	50	15.0 (H)	97
40% Alcohol extract of acetone dried powder	10.0	1700	20.4	66
	2.5	400	14.3	16
0.5 SAS fraction (see Paper II, procedure 4)	50.00	50000	34.6	181
	25.00	25000	23.8	94
	25.00	25000	29.5 (H)	275
	10.00	10000	20.0	63
	10.00	10000	22.5 (H)	196
	2.50	2500	15.0	22
	1.00	1000	17.8	45
	1.00	1000	14.0 (H)	84
	0.50	500	14.6	19
	0.25	250	14.4	17
	0.10	100	14.6	19
	0.05	50	13.6	11
	0.02	20	12.4	—
ICSH fraction *L* 79 C	5.00	50000	36.2	194
	1.00	10000	21.2	72
	0.50	5000	20.4	66
	0.05	500	20.0	63
L 141 *DTP*[3]	5.00	50000	19.0	55
	0.50	5000	13.0	6
	0.05	500	14.0	14
Controls		92 rats	12.3	
		19 rats	7.6	
Acetone dried glands, alkaline extracts[2]	—	1000	52	420
	—	500	38	280
		250	31	210
L 141 *DTP*[3] (ICSH)	2.00	20000	47	370
	1.00	10000	38	280
	0.50	5000	28	180
	0.25	2500	22	120
	0.12	1200	18	80

[1] Normal rats unless marked by (H) which signifies hypophysectomized rats (28–30 days at operation, 6–8 days post-operative at onset of injections.)

[2] To correlate these results of subcutaneous injections with results from implantation and injection procedures of other authors, this fraction was also injected at the same level (22 mg.) (*a*) intramuscularly and (*b*) over a 6-day period with autopsies on the 8th day. Both these assays gave slightly smaller effects in hypophysectomized rats. (*a*): 13.3 mg. S.V. (=75%); (*b*) 11.8 mg. S.V. (=55%).

[3] Assays by Dr. H. L. Fevold of our preparations.

Effects of ICSH in Female Rats

a) Alone. Purified ICSH causes repair of the ovarian interstitial tissue of the immature hypophysectomized rat (26 to 28 days at operation, 6 to 8 days postoperative), when a total of 0.005 to 0.01 mg. is injected once daily intraperitoneally for 3 days with autopsies 24 hours after the last injection. The amount of protein giving this response has been defined as 1 ICSH unit.[2] Three mg. of the ICSH (300 U) does not yet cause any histologically detectable follicular stimulation in such rats on subcutaneous injection. On injection into normal immature rats in the same manner ICSH has no effect on ovaries or uterus at levels below 1 to 2 mg. Corpora lutea begin to appear at these levels.

b) Antagonism against PMS. When injected intraperitoneally in normal immature female rats in combination with a standard amount of pregnant mare serum gonadotropin (PMS), 0.01 mg. of the ICSH decreases the ovarian weights produced by the PMS by 25% and higher doses decrease it by as much as 70%. Similar antagonistic effects can be observed against chorionic and menopause urine gonadotropin but not against FSH fractions from the pituitary. No significant antagonism of purified ICSH against any other gonadotropin could be demonstrated in hypophysectomized rats. (For a detailed account of the properties of antagonism inherent in pituitary ICSH and FSH fractions, cf. 6.)

c) Augmentation in combination with FSH. Many experiments have been performed studying the effect of subcutaneous injection of ICSH in conjunction with FSH either mixed or at different sites of the body, in normal and hypophysectomized rats (tables 1, 2). As stated before, ovarian weights are increased in both types of rats to about double those given by the FSH alone at certain levels and not at others. Corpora lutea similarly appear in normal rats at certain levels only, not always coinciding with the levels at which maximal ovarian weight augmentation is observed. In hypophysectomized rats luteinization is observed only at the higher levels (10 to 25 U ICSH in conjunction with the standard amount of FSH).

In both types of rats low doses of ICSH in conjunction with FSH cause pronounced uterine development; thus in hypophysectomized rats estrous uteri were regularly found at autopsy after 5 daily subcutaneous injections (at different sites of the body) of 2.5 U FSH (0.01 mg.) and 0.5 U of ICSH (0.005 mg.), an effect which was observed only in a few instances when the FSH alone was injected. This effect thus represents the most sensitive method for the detection of ICSH.

Effects of ICSH in Male Rats

For assays in male rats, groups of 5 animals were employed, either normal immature (22 to 23 days old) or hypophysectomized (28 to 30 days at operation, 6 to 8 days postoperative). The normal rats were injected for 5 days and autopsied 24 hours after the last injection, the hypophysectomized rats generally for the same time period, in a few instances for 6 days with autopsies on the 8th. The subcutaneous rather than intraperitoneal route of injection was employed as a rule, although no significant difference between the two was observed in male rats. The weights of seminal vesicles (plus coagulatory gland) were taken in all instances, weights of prostate and testis occasionally (table 3).

On injection of manifold pituitary extracts and fractions in all stages of purification, the seminal vesicles were always only slightly increased. Although levels as

[2] 2.5 International Units of pregnancy urine gonadotropin were found to give the same effect (repair of the interstitial tissue) under the same conditions (see table 5).

TABLE 4. FAILURE OF FSH TO AUGMENT THE EFFECT OF PITUITARY ICSH ON SEMINAL VESICLE WEIGHTS OF MALE RATS

Total Dose		Average seminal vesicle weights[2]	% Increase
FSH	ICSH		
R.U.[1] (5V13B) (1U=0.025 mg.)	(L57AB) (1U=0.01 mg.)	mg.	
—	100	15[3]	22
—	20	15	22
—	5	12	0
20	—	15	22
20	100	19	54
20	20	18	46
20	5	15	22
10	—	12	0
10	100	20	63
10	20	17	38
10	5	16	30
4	—	11	0
4	100	20	63
4	20	19	54
4	5	15	22
2	—	14	14
2	100	19[3]	54
2	20	16	30
2	5	18	46
(IIF43D) (1U=0.02 mg.)	(IIF43C1)[4] (1U=0.02 mg.)		
—	50	21	71
—	10	16	30
—	5	16	30
5	—	13	6
5	45	17	38
5	5	16	30
(IIF36A) (1U=0.01 mg.)	(L65B) (1U=0.01 mg.)		
—	100	21	71
5	—	11	0
5	100	19	54
—	5	9 (H)	18
5	—	9 (H)	18
5	5	10 (H)	32
(VS15B) (1U=0.025 mg.)	(VS15A)[4] (1U=0.025 mg.)		
—	120	9 (H)	18
60	—	9 (H)	18
60	120	10 (H)	32

[1] Female hypophysectomized R.U. as defined on table 1.
[2] Normal rats unless marked by (H), i.e. hypophysectomized rats.
[3] 4 rats per group instead of the usual 5.
[4] Crude ICSH fractions (0.5 SAS fraction).

high as 5 to 50 mg. of purified preparations were at times employed, corresponding to 500 to 2000 ICSH U, the seminal vesicle weight increases exceeded 100% only in 5 groups of rats out of almost 100 injected groups.[3] The dose response curve for crude as well as purified pituitary fractions was found to be so flat that no quantitative conclusions could be drawn by this method of assay, unless a very great number of rats were used per group. Neither could significantly greater effects on the accessories in the male be observed after simultaneous injection of FSH with the ICSH (table 4). The seminal vesicles in hypophysectomoized rats seemed only slightly more responsive than in normals and the prostate in both types of rats reacted similarly to the seminal vesicles.

DISCUSSION

Criticism has attended the proposal of the designation, interstitial cell stimulating hormone (ICSH) for this substance, (1) but defense is readily afforded by a surveyal of the recorded effects of ICSH on our rats.

It has been shown that ICSH resembles 'LH' in giving some ovarian weight augmentation on combination with FSH and some development of the accessories of the male rat. It has become evident, however, that these effects are given only to a very limited degree. Thus a maximal ovarian weight augmentation of up to 100% was produced by 10 to 20 U of ICSH in combination with a constant amount of FSH, whereas higher levels generally produced smaller effects. Similarly, in the male, excessive doses of the hormone are needed to cause increases in accessory weights exceeding 100% in a 5 to 7-day assay period. It is consequently not possible to measure the 'LH' content of ICSH in terms of the units defined by Fevold; the conclusion would be that ICSH is almost free from 'LH'.

An attempt was therefore made to isolate from sheep pituitaries a hormone showing the properties of 'LH' following Fevold's procedure as well as others. The outcome of extensive work of this type has always been negative. Neither injection of suspensions of thoroughly ground fresh or stored sheep glands nor of any extract prepared from these nor from any fractions obtained from such extracts by chemical means gave 'LH' effects exceeding those obtained with purified ICSH, even though very great doses were at times employed (2.5 gm. pituitary tissue and 50 gm. tissue equivalents of chemical fractions). As will be shown in Paper III of this series, about 0.8 gm. pituitary tissue was found to contain one 'LH' U, as measured by its effect on seminal vesicle weights of the Long-Evans strain of rats, while only 0.015 or 0.009 gm. tissue, respectively, were needed to cause follicular stimulation or interstitial tissue repair in the female hypophysectomized rat. These facts are in sharp contrast to Fevold's finding that sheep glands contain 2 to 4 times as many seminal vesicle stimulating as ovary stimulating units (1).[4] We thereupon investigated sheep pituitaries from other than the usual source (lambs, 5 to 6 months old) and also glands from older sheep, but found neither of these to give effects on the male accessories exceeding 100% (see Paper III, table 1).

It hence appeared that a difference in the responsiveness of the rat material of different laboratories to pituitary ICSH fractions would afford the only explanation of such results. We therefore sent Dr. Fevold a crude pituitary powder as well as a purified

[3] The injection of purified fractions in suspension as tannates doubles their effects on seminal vesicle weights in our normal and hypophysectomized rats. Increases of 100 to 200% were thus produced by 1 or 10 mg. protein tannate respectively of the 0.5 SAS fraction.

[4] It also stood in contrast to our observation that by 5 daily implantations of a total of 0.2 gm. of fresh sheep glands great increases in the accessories (400%) could be produced in our rats. The interesting findings which followed and led to a clarification of this question will be discussed in Paper III.

ICSH preparation in exchange for his unfractionated extract.[5] As we had suspected, upon assay in our rats, Dr. Fevold's extract proved to contain very little 'LH' (4 male R.U., as defined and assayed by this author giving only about 30% increase in seminal vesicle weights of Long-Evans rats), while both our preparations when assayed in the rats of the Harvard laboratory gave increases in seminal vesicle weights reaching 400%, as characteristic for 'LH'[6] (table 3).

Thus the different properties ascribed to LH and ICSH by the two laboratories

TABLE 5. COMPARISON OF THE RELATIVE POTENCY OF DIFFERENT GONADOTROPHINS

Relative Potency is Expressed as Multiples of Minimal Effective Dose in Normal Immature Female Rats (MED) Necessary to Elicit Different Gonadotrophic Effects

Effect	Type of rats[1]	Human pregnancy urine gonadotr.[2]	Pregnant mare serum gonadotr.[3]	Non-pregnant serum gonadotr.[4] (M.E.D. = 25 mg.)	Normal male urine gonadotr.		ICSH from pituitary extracts
					Prospermin (M.E.D. = 1 mg.)	Alcohol precipitate (M.E.D. = 0.7 mg.)	
Interstitial tissue repair	(Hypophysectomized ♀)	2 to 3	2 to 4	2	2 to 3	4	0.01
Follicular development	(Hypophysectomized ♀)	(>300)	6 to 10	10	10	4	(>2)
Corpus luteum formation	(Hypophysectomized ♀)	(>300)	10 to 20	(>10)	20	(>10)	(>2)
Corpus luteum formation	(Normal ♀)	8	2 to 4	(>2)	5 to 10	(>2)	1
100% Incr. in seminal vesicle weights	(Normal ♂)	5	6		4	(>4)	ca 1
	(Hypophysectomized ♂)	4	5			(>4)	ca 1

[1] Injections were performed by the most advantageous route for any given hormone or effect, i.e., intraperitoneal for serum gonadotropins and for interstitial tissue repair and corpus luteum formation in general,—otherwise subcutaneous.

[2] These ratios were found to be the same for the International Standard Preparation for human pregnancy urine gonadotropin with an M.E.D. in normal rats of 0.1 mg. and for purified commercial preparations (follutein, antuitrin-S, etc.) with M.E.D.'s as low as 0.005 mg.

[3] These ratios were found to be the same for the International Standard Pregnant Mare Serum Gonadotropin with an M.E.D. in normal rats of 0.25 mg. and for highly purified preparations kindly put at our disposal by Dr. H. H. Cole, Dr. E. Schwenk and D. Wonder, with M.E.D.'s ranging from 0.00025 mg. to 0.001 mg.

[4] Prepared according to (8).

appear to be due to difference in the responsiveness of rat strains to one and the same substance. The limited responsiveness of the Long-Evans strain of rats of either sex to pituitary ICSH fractions stands in contrast to their ability to respond readily to gonadotropins from pregnancy as well as normal men's urine and from the serum of pregnant or non-pregnant mares.[7]

[5] We wish to express our grateful appreciation for Dr. Fevold's cooperation in this respect.

[6] From the fact that our pure ICSH fraction (*L 141 DTP*) caused seminal vesicle weight increases proportional to the doses administered (up to 370%) when assayed by Dr. Fevold, this author concluded that FSH was present in this preparation. We would regard it as preferable to base such a decision on the accepted test methods for FSH (follicular development in the female and tubular development in the male rat), according to which ICSH is free from FSH at the highest levels tested.

[7] We are indebted to Mr. D. Wonder and the Cutter Laboratories for supplying us with serum of non-pregnant mares, verifying the non-pregnancy by autopsy, and cooperating with us in the isolation of the hormone.

As illustrated in table 5, only 1 to 2 ICSH U of these gonadotropins from urine and serum corresponded to 1 'LH' unit (as assayed in male rats), in contradistinction to pituitary extracts of which 100 ICSH U are needed to cause 100% increase in seminal vesicle weights. Similarly in the female all these non-pituitary preparations differ from pituitary ICSH in that they show striking 'augmentation' of ovarian weights; those with follicle stimulating activity like pregnant mare serum and male urine gonadotropin produce ovaries weighing over 100 mg. on injection of only a few multiples of the minimal dose; and pregnancy urine gonadotropin which is free from FSH shows similar striking effects upon combination with FSH.

These observations led to a further comparative study of different gonadotropic effects given by these gonadotropins (table 5). It became evident that all urine and serum preparations gave detectable gonadotropic effects—follicular and uterine stimulation—at lower levels in normal immature than in hypophysectomized rats, differing in this respect from predominantly follicle stimulating gonadotropins (from hypophysis or menopause urine). It also became evident that the same multiple, i.e., a 2 to 4-fold of the minimal dose in normal rats, of all these preparations represented their minimal dose for repair of the interstitial tissue of the hypophysectomized rat. From the fact that this ratio was constant for all non-pituitary gonadotropins with ICSH properties, while their FSH content varied considerably, it appears very likely that the first effect caused by each of these preparations in normal rats is due to a stimulation of the rat's own pituitary by the ICSH-like component. In contrast to this, ICSH isolated from the pituitary is a hundred times less effective in normal rats than in hypophysectomized rats, and consequently it appears that this hormone has only very slight stimulating effect on the pituitary.

All urinary and serum gonadotropins have yet another feature in common which differentiates them from all pituitary fractions: they give no antagonistic effects under conditions favorable for the detection of antagonism in all pituitary fractions.

It must be noted that in all the respects enumerated above even those urine and serum gonadotropins which are most likely of pituitary origin were found to differ from ICSH of pituitary extracts. A new point of view was suggested by such findings for the classification of gonadotropins, i.e., secreted hormones since they can be observed in the blood stream and in the urine, as contrasted to the hormones extracted from the pituitary gland by chemical means. The antagonistic properties of the gonadotropins of the latter type as well as their ineffectiveness in certain respects differentiates them, and in particular the ICSH, clearly from what we believe to be the secreted gonadotropins of pituitary origin, i.e., those found in the urine and serum of healthy non-pregnant human beings and animals.

It appears possible that some of the extracted hormones, e.g., the ICSH, may represent a precursor of the actual secreted hormone. On the basis of this working hypothesis the different responsiveness of different strains of rats to pituitary fractions might be explained by the assumption that some rats are able to liberate the active hormone from the injected precursor and thus react in similar manner to both types of gonadotropins, while the Long-Evans strain does not possess such a mechanism.

A further discussion of other aspects of this precursor theory in connection with experimental work of a different nature will follow in Paper III.

SUMMARY

The biological effects of pure ICSH in male and female immature rats, both normal and hypophysectomized, have been described. This hormone, as well as crude pituitary extracts, when assayed in the Long-Evans strain of rats according to the methods proposed for 'LH' proved very ineffective in increasing male accessory weights or in

causing ovarian weight augmentation in combination with FSH; however, with the kind cooperation of Dr. Fevold it was possible to show that crude pituitary extracts as well as pure ICSH cause great seminal vesicle weight increases in another rat strain while preparations stated to be rich in 'LH' fail to do so in Long-Evans rats.

Assays of various urine and serum gonadotropins in the Long-Evans strain of rats have shown all these preparations (with the exception of the gonadotropin from menopause urine) to produce at low doses the effects characteristic for 'LH', i.e., pronounced increase in the weights of accessories in male rats and in ovarian weights in combination with FSH in females. This was found to be true not only for gonadotropins of chorionic origin but also for those which are in all probability secreted by the pituitary. A working hypothesis has been proposed to explain the difference in the gonadotropic effects of pituitary extracts in different strains of rats.

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INTERSTITIAL CELL STIMULATING HORMONE. II. METHOD OF PREPARATION AND SOME PHYSICO-CHEMICAL STUDIES[1]

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A RECENT PUBLICATION (1) from this laboratory described a chemical procedure to separate two gonadotropic fractions from sheep pituitary: the follicle stimulating hormone (FSH) and the interstitial cell stimulating hormone (ICSH). By modification of this method it has now been possible to secure the ICSH in a homogeneous state as judged by electrophoresis experiments. The biological properties of the preparation have been studied in detail and results appeared in Paper I (2). The method of purification and some of the physicochemical properties of the product are presented here.

The method of purification is given below; all steps were carried out at 4 to 6°C.

(*a*) The 40% alcohol extract (1) of acetone-dried sheep pituitaries was used as the starting material. The extract was precipitated with alcohol and dried with absolute alcohol and ether.

(*b*) Fifty grams of this powder were extracted with 3 liters of water. The extract was brought to about PH 4.5 with the aid of 5 N acetic acid and was precipitated with an equal volume of acetone.

(*c*) The precipitate was extracted with one liter of 1% saline solution and insoluble material was discarded.

(*d*) The 1% saline extract was brought to 0.5 saturation with SAS.[2] The precipitate obtained was reprecipitated twice more, under similar conditions, keeping the protein concentration approximately the same. (The volume was therefore decreased about 70% in the 2 steps.) The precipitate was removed by centrifugation and the supernatant liquid saved for the purification of FSH.

(*e*) The 0.5 SAS precipitate was dissolved in water so that the final volume was 800 cc., and 100 cc. of SAS was added. The 0.2 SAS precipitate contained very little activity and was discarded. The supernatant liquid[3] was brought to 0.4 SAS by addition of more SAS. This procedure was repeated twice more in decreasing volume (the volume during final precipitation was 500 cc.).

(*f*) The final 0.4 SAS precipitate was dissolved in 300 cc. solution and brought to 0.37 SAS by addition of 175 cc. SAS, centrifuged,[4] and SAS was added to the supernatant to 0.4 saturation.

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[2] SAS = saturated ammonium sulfate.
[3] The hydrogen ion concentration of the supernatant liquid was measured with glass electrode and found in every case to be PH 5.3 to 5.4. Therefore, the whole procedure of purification is actually conducted in a solution of PH 5.3 to 5.4.
[4] The 0.37 SAS precipitate contains somewhat more inert globulin than the supernatant but is sufficiently valuable to save for biological studies. It was washed twice with 100 cc. of 0.37 SAS solution.

(g) The 0.4 SAS precipitate was again dissolved in water to a volume of 150 cc. and the process (f) was repeated twice. At this stage of purification the preparation regularly had an M.E.D. for ICSH of 0.01 mg.; injected subcutaneously at the 1.0 mg. level and above it caused no follicular development in hypophysectomized rats.

(h) To remove the last trace of FSH contaminating the fraction precipitating between 0.37 and 0.4 SAS, it was dissolved in water (1% protein) and brought to 2.5% trichloroacetic acid by addition of 10% solution. The precipitate formed was dissolved in a small volume of alkaline solution and the trichloroacetic acid precipitation was repeated. The final precipitate was dissolved in a small amount of water by the addition of a few drops of N NaOH. The clear solution was dialyzed against distilled water for a few days. During the dialysis there is usually some precipitate formed. Since the potency of this water insoluble material is the same as that of the soluble substance, it was customary not to centrifuge it off. The ICSH was obtained in dry form by evaporation of the frozen block in vacuum.

The ICSH thus obtained represents the highest purification thus far obtained. It does not stimulate follicular development in hypophysectomized rats at the highest dose tested, 3.0 mg. Its minimum effective dose for interstitial tissue repair is between 0.005 and 0.010 mg. Thus approximately 300 to 600 times the M.E.D. for ICSH can be given subcutaneously to hypophysectomized animals before the development of follicles and formation of corpora lutea (fig. 2). Table 1 summarizes the yield and potency from a number of experiments. The last column gives the ratio of unitage of ICSH to that of FSH at different stages of purification.

TABLE 1. YIELD AND POTENCY OF ICSH PREPARATION FROM SHEEP PITUITARY

Material	Weight	ICSH[1]	FSH[2]	Ratio
	gm.	R.U./gm.	R.U./gm.	
Frozen fresh	4,000.0	110	67	1.7
Acetone dried	1,200.0	1,000	400	2.5
40% Alcohol powder	50.0	20,000	4,000	5.0
0.5 SAS ppt.	7.0	20,000	2,000	10.0
Purified ICSH fraction	0.5	100,000–200,000	350	300–600

[1] R.U. ICSH is the minimum dose administered intraperitoneally (conditions as above) which causes interstitial tissue repair.

[2] One R.U. of FSH is the minimal amount which, given subcutaneously in 3 daily doses, followed by autopsy 72 hr. after first injection, causes resorption of follicular growth in hypophysectomized female rats (26 to 28 days at operation, 6 to 8 days p.o. at autopsy).

The degree of purity of this ICSH preparation has been demonstrated further by biological tests for other hormones. The preparations were free of contamination with FSH, thyrotropic, lactogenic, adrenotropic and growth hormones, when tested in the doses stated below, which in all cases were much larger than those necessary to produce the specific effects. The local crop test for lactogenic hormone was negative at 0.68 mg.[5] No thyrotropic activity could be detected at 0.5 mg.[6] by the 1-day-old chick thyroid weight test. No growth promoting activity or adrenotropic activity

The precipitate was then dissolved in a small amount of water and dialyzed against distilled water until free from electrolytes. A dry powder was obtained by evaporating the frozen solution. This fraction shows no FSH activity when injected in 2.0 mg. doses subcutaneously in hypophysectomized rats, and has an M.E.D. for interstitial cell repair of 0.01 mg. As indicated by electrophoretic studies, this fraction always contains a low concentration of another component which presumably is an inert globulin. Therefore, this fraction cannot be considered to be pure ICSH.

[5] 0.68 mg. represents 68 ICSH U; the M.E.D. (systemic and unit) for the purest lactogenic hormone is 0.0001 mg.

[6] Five tenths mg. represents 50 ICSH U; the M.E.D. (chick test) for purest thyrotropic hormone is 0.007 mg.

was detected in hypophysectomized rats when injected at a total dose of 8 mg.[7] in 10 days.

A high degree of purity was also indicated by the diverse chemical methods applied without success in efforts to increase its potency. Repetition of the ammonium sulphate and trichloroacetic acid precipitation still yielded a product containing 100 to 200 R.U. per mg. At the point of maximum precipitation, the supernatant still had the same potency per mg. of protein. As already stated in procedure (*h*), the ma-

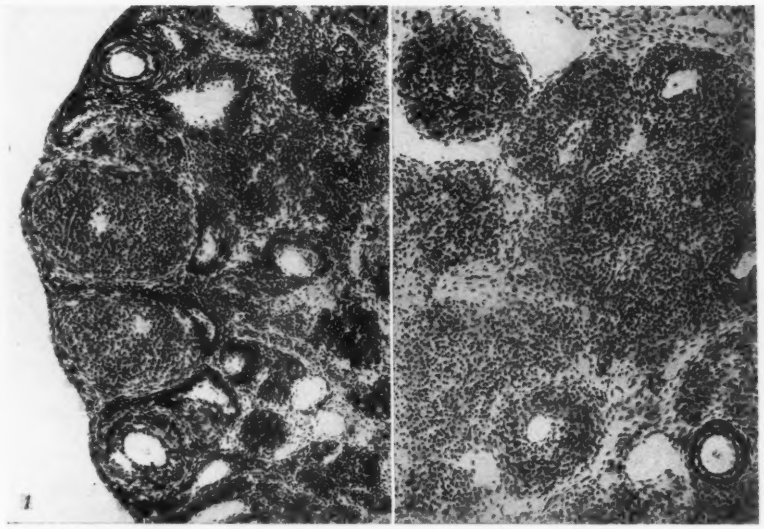

Fig. 1. OVARY OF CONTROL HYPOPHYSECTOMIZED RAT; AGE AT OPERATION 27 DAYS, AT AUTOPSY 38 DAYS; 2 OVARIES = 12 mg.

Fig. 2. OVARY OF HYPOPHYSECTOMIZED RAT INJECTED WITH A HOMOGENEOUS ICSH (*L 141 DTP*, minimum dose 0.005–0.010 mg.). Total dose (subcutaneous) 2 mg. Age at operation 27 days, at onset of injection 35 days, at autopsy 38 days. 2 ovaries = 27 mg.

terial separating on dialysis had the same potency as the soluble substance. All these findings suggest that the preparation may be a pure protein. The preparations have therefore been investigated in the Tiselius apparatus to confirm their homogeneity. As shown in figure 3, only one sharp Schlieren boundary was observed after passing the current through the solution for 90 minutes. The mobility of the preparation was found to be 6.36×10^{-5} cm^2/volt/sec. in phosphate buffer of pH 7.53 and ionic strength 0.05 at 1.5°C. A more detailed report on the electrophoresis experiments together with its osmotic behavior will be described in a subsequent paper.

Carbyhydrate content. The presence of carbohydrate in ICSH fractions has already been reported in a previous publication (3) where it was shown that the two gonadotropic factors (FSH and ICSH) have different sugar and glucosamine contents; FSH fractions were always found to have a higher value than ICSH. As it is well known that a high sugar content in a protein makes it less precipitable or non-precipitable by trichloroacetic acid, advantage was taken of this characteristic to free ICSH from

[7] Eight mg. represents 800 ICSH U, the M.E.D. (hypophysectomized rat test) for the purest growth hormone is about 0.01 mg.; the minimum dose for detectable repair of the adrenals of hypophysectomized rats in 10 days given by the best adrenotrophic hormone is less than 0.2 mg.

FSH. The measure of success of this method is shown in table 2; fractions *L* 61 C and *L* 141 C are purified ICSH preparations but contain some FSH (M.E.D. for FSH, 0.5 to 1.0 mg.). After treatment with trichloroacetic acid the carbohydrate content of the precipitate decreased while the supernatant liquid contained a correspondingly higher content of sugar.

Fig. 3. SCHLIEREN PHOTOGRAPH OF THE ELECTROPHORESIS OF 0.6% SOLUTION OF PITUITARY INTERSTITIAL CELL STIMULATING HORMONE. The exposure was taken after passing current for 90 minutes. Boundary migrated to the anode.

TABLE 2. CARBOHYDRATE CONTENT OF FRACTIONS SEPARATED FROM THE 0.37 TO 0.4 SAS FRACTION BY TRICHLOROACETIC ACID

Fraction	*L* 61 C	*L* 141 C
% Protein precipitable by trichloroacetic acid	50.0	52.0
% Mannose in starting material	5.40	6.04
% Mannose in trichloroacetic acid ppt.	4.20	4.30
% Mannose in supernatant	6.2	—

The orcinol method as modified by Sørensen and Haugaard (4) was used for the sugar determinations. Sorensen and Haugaard showed characteristic differences in the behavior of the individual hexoses by this method. They found it possible to identify individual hexoses in proteins. Using the same technic, the conclusion was reached that the sugar in the ICSH fraction is probably mannose. This naturally must be confirmed by isolation of this sugar from the hormone. Table 3 summarizes the ratios of the extinction coefficients E_G/E_B[8] determined for the sugars, the mixture of sugars and the hormone.

TABLE 3. RATIO OF EXTINCTION COEFFICIENT OF ICSH AND VARIOUS HEXOSES[1] IN ORCINOL REACTION

Preparation	E_G/E_B
Mannose	5.71
Galactose	3.95
Glucose	4.50
ICSH	5.56
Glucose-Mannose	4.68
Glucose-Galactose	4.46
Galactose-Mannose	4.70

[1] Concentration of sugar = 0.2 mg/cc.

[8] E_B refers to the negative logarithm of the transmission where the blue filter is used in the Cenco photolometer; E_G refers to the use of the green filter.

It is of interest that both human pregnancy urine (5) and pregnant mare serum (6) gonadrotropins contain galactose while pituitary ICSH contains mannose. The sugar content is also higher in the former group than in pituitary ICSH (Table 4). It should be noted that the molar ratio of mannose to glucosamine in ICSH was approximately 1:1; suggesting that the polysaccharide is probably a polymerized complex of glucosamine-manose.

TABLE 4. SUGAR AND GLUCOSAMINE CONTENT OF PREGNANCY URINE GONADOTROPIN, EQUINE SERUM GONADOTROPIN AND ICSH

Hormone	Nature of sugar	Content	
		Hexose	Glucosamine
		%	%
ICSH	Mannose	4.45	5.85[1]
Prolan (ref. 5)	Galactose	10.70	5.20
PMS (ref. 6)	Galactose	14.10	12.80[1]

[1] We thank Dr. H. Fraenkel-Conrat for these analyses.

Chemical characteristics. The fact that the homogeneous preparations of ICSH give positive biuret, xanthoproteic, Millon and ninhydrin reactions indicates that ICSH is a protein. The hormone has a maximum precipitation (about 40%) at approximately PH 4.5; this is probably close to the isoelectric point.[9]

The preparations contain 14.2% of nitrogen and 0.49% of amino nitrogen as determined by van Slyke gasometric analysis. It gave the test for labile sulphur but no phosphorus was found.

Action of ketene. As has already been reported in Paper I, these purified preparations which stimulate interstitial cells of hypophysectomized rats have another biological action: namely, to antagonize the effect of certain non-pituitary gonadotropins in normal rats. The question whether these two biological actions are caused by separate hormones or are characteristic of a single substance is not settled. The evidence given by electrophoresis experiments that the preparation consists of a single protein may not be sufficient as it is possible that a second substance, 'the antagonist,' has the same mobility as the ICSH.

TABLE 5. EFFECT OF KETENE ON POTENCY OF ICSH

Time of acetylation at 20–21°C	Interstitial cell stimulating action	Antagonistic action
min.	R.U./mg.	R.U./mg.
0	100	100
5	<10	<10

Interpretations made from the action of certain specific chemical reagents might adduce additional evidence regarding this question. Ketene is well known to be a useful and specific acetylating reagent for protein. It reacts very rapidly with free amino groups without affecting any other groups in the molecule. By use of this reagent it has already been possible to show (7) that the physiological activity of the pituitary follicle stimulating and interstitial cell stimulating hormones and of the gonadotropic substance in pregnant mare serum is dependent on the free amino groups. The same method shows that these groups are not essential for human pregnancy urine gonadotropin. In the preceding publication the effect of ketene on the 'antagonism' shown by ICSH preparations was not reported; therefore, the purest preparations herein described have been reinvestigated with particular reference to this point.

[9] Preliminary electrophoresis experiments indicate the isoelectric point at PH 4.60 to 4.80.

The protein to be ketenized was dissolved in pH 5.7 M acetate buffer. The solution, containing 1 mg. per cc. of protein, was allowed to react with ketene[10] at room temperature. The biological assay of the solution (after dialysis) showed that both the interstitial cell stimulating and antagonistic potencies had been greatly reduced after treatment for 5 minutes. It is therefore apparent that the free amino groups are responsible for both biological properties. It should be recalled (9) that these properties are also lost on cysteine treatment.

SUMMARY

The preparation of a highly purified globulin from sheep pituitary extracts having two biological effects (interstitial cell repairing and antagonistic) has been described.

The protein contains 4.45% hexose. The use of the orcinol reaction indicates that the hexose is not galactose or glucose; it is probably mannose. The preparation also contains glucosamine; the molar ratio of mannose to glucosamine is approximately 1:1.

Judged by ketene treatment free amino groups appear essential for both interstitial cell stimulating and 'antagonistic' properties of the protein. Electrophoresis experiments also indicate that the preparation is a single substance. There is no evidence from these studies that the 'antagonism' shown by these preparations is caused by a substance separate from that causing interstitial cell repair.

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ADDENDUM

While this paper was in press, an article appeared by Shedlovsky et al. (10) in which the claim is made to "the isolation in pure form of the interstitial cell stimulating (luteinizing) hormone of the anterior lobe of the pituitary gland." The product obtained by these authors differs considerably from our pure protein in its physico-chemical properties. These differences may be due to any of the following facts (*a*) We used sheep glands; the above authors used swine glands. (*b*) The methods of preparation differ widely in the early stages. Either of these may have resulted in a change of the hormone molecule. (*c*) The pure protein of either laboratory may represent an inert protein, contaminated with physico-chemically undetectable amounts of the actual hormone. (*d*) The new method of assay employed by the above authors makes use of another criterion than ours, based on maintenance, rather than repair of the interstitital tissue. The two assay methods have not yet been quantitatively correlated.

An inter-exchange of the pure fractions of the two laboratories as it is planned, will throw more light on this question. Preliminary assays seem to indicate that the two assay methods are of comparable sensitivity and the pure proteins of similar activity.

[10] Ketene is obtained by an improved generator of Herriott type (8).

INTERSTITIAL CELL STIMULATING HORMONE III. METHODS OF ESTIMATING THE HORMONAL CONTENT OF PITUITARIES[1]

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IN THE PRECEDING PAPER on the biological properties of ICSH it has been demonstrated that this protein neither increases considerably the weights of the accessories in the male rat nor produces ovarian weight 'augmentation' in the female rat to any great extent. Nor were great effects of this kind obtained by injection of diverse pituitary extracts nor of any fraction prepared from such crude extracts.

Since implantation of fresh glandular tissue is often regarded as the standard method for the evaluation of the hormonal content of the pituitary and other endocrines, this technic was then resorted to.

METHOD

Heads of sheep were brought to the laboratory immediately after the death of the animals. The pituitaries were dissected under aseptic conditions, within 1 to 3 hours after death, minced with scissors, the desired amount of tissue weighed, suspended in Locke solution, and immediately injected by means of a glass cannule into the leg muscle of rats. This was done daily for 5 days in the case of male and 3 days in the case of female recipients, with autopsies 24 hours after the last injection. Groups of 5 males or 3 females were used unless otherwise specified.

RESULTS

On implantation of fresh sheep anterior pituitaries at several levels ranging from 10 to 200 mg. (total dose) into hypophysectomized or normal immature male rats, the seminal vesicles were found increased in weight from 100% to over 400% (table 1; 1, 2*a*, 3*a*, 4*a*). As shown in Paper I, responses such as the latter have never been approached by injection of extracts or suspensions of stored glands, even though equivalents of much more tissue were at times employed. The importance of the freshness of the glands was also demonstrated when these were implanted 30 hours after the death of the sheep; 200 mg. caused then no more than 100% increase in seminal vesicles (table 1, 5). Also, stored frozen glands from another than the usual source (table 1, 6) and from another type of sheep (2-year-old females instead of the usual lambs) (table 1, 7) were found of low activity. When the effect of thorough grinding of fresh glands with sand in Locke solution before implantation was investigated,

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such suspensions were found slightly more effective than stored glands but considerably less potent than fresh minced gland implants (table 1, 8).

The effect of freezing fresh sheep pituitaries before implantation was then investigated. Fresh glands were treated 3 to 4 times with liquid air with intermittent thawing and then implanted in the usual manner and within the usual time after the death of the sheep into immature normal and hypophysectomized male rats. Glands

TABLE 1. EFFECT OF IMPLANTATION OF FRESH STORED, FROZEN OR GROUND[1] SHEEP ANTERIOR PITUITARIES IN MALE RATS

No. of expt.	Type and treatment of implanted glands	Total dose	Type of rat[2]	Seminal vesicles weight[3]	Incr.	Prostate weight	Incr.	Testes weights	Incr.
		mg./rat		*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
1	Fresh	200	H	42	450	134	270	501	174
		100	H	34	350	122	240	455	149
		50	H	20	163	82	128	350	91
		25	H	17	124	82	128	364	99
		10	H	15	97	67	86	390	113
2	a: Fresh	200	N	48	290	168	133	600	26
	b: Frozen	200	N	18	46	111	54	546	15
3	a: Fresh	200	N	51	315	193	168	786	65
	b: Frozen	200	N	21	71	132	83	666	40
4	a: Fresh	200	H	31	308	103	186	418	128
	b: Frozen	200	H	15	97	64	78	436	138
5	Stored	200	N	22	79				
6	Frozen (Swift & Co.)	200	N	24	94	134	86	605	27
7	Glands of mature sheep frozen	200	N	15	22			470	—
8	Ground	200	N	31	152	158	120	571	20
9	Frozen	1000	N	32	160	175	143	742	56
	Controls		H	7.6 (Av. 19 rats)		36 (Av. 10 rats)		183 (Av. 10 rats)	
			N	12.3 (Av. 92 rats)		72 (Av. 29 rats)		477 (Av. 27 rats)	

[1] Fresh: Pituitaries are minced and implanted in suspension in Locke solution immediately after removal from sheep heads and within 4 hours after the death of the animal.

Store: Pituitaries are kept in an atmosphere saturated with toluene at +3°C. for 24 hours.

Frozen: Pituitaries immediately after removal from the heads are frozen 4 times with liquid air, with intermittent thawing; they are implanted within 4 hours after the death of the animal.

Ground: Fresh pituitaries are ground thoroughly with sand and injected intramuscularly in suspension in Locke solution within 4 hours after the death of the animal.

[2] N=normal immature rats, 22 to 23 days at onset; one-fifth of the total dose implanted intramuscularly daily for 5 days; autopsies 24 hours after the last implantation.

H=hypophysectomized rats, 28 to 30 days at operation, 6 to 8 days post-operative, implanted and autopsied as above (5 rats per group).

[3] Two seminal vesicles plus coagulatory glands.

which remained in the sheep heads during this time were implanted unfrozen for the purpose of comparison (table 1; 2, 3, 4). In every case 200 mg. of the fresh glands caused increases of 300 to 400% in seminal vesicle weights while the same amount of the frozen glands produced up to 100% increase and a 5-fold of this dose did not lead to a much greater response (table 1, 9). The same difference in the effect of implantation of fresh and frozen glands was observed to a slightly lesser degree in regard to

TABLE 2. EFFECT OF IMPLANTATION OF FRESH, STORED, FROZEN OR GROUND SHEEP ANTERIOR PITUITARIES IN FEMALE RATS

Total dose	Fresh Minced Glands				Frozen, Stored or Ground Glands[2]			
	Ovaries		Uterus		Ovaries		Uterus	
	Weight	Description	Weight	Description	Weight	Description	Weight	Description
			A. Normal Rats[1]					
mg./rat	mg.		mg.		mg.		mg.	
120	A[3] 149 72 103 D 39 80 68 51	lF, CL, do, db lF, CL mlF, CL smF, CL lF, cysts, CL m, lF, CL, do	137 127 215 163 147 121 118	ml est l l l ml	A 43 37 20 D 16 28 18 26	Frozen sF, sev. CL lF sF, sev. CL s, few mF smF mF, 2 CL	80 83 54 63 47 65 47	m m m s sm s
90	E 64 46 68 83 78	CL, do lF, CL lF, CL lF, CL, do	134 106 133 162 128	l m l l	E 33 27 29 31 23	Stored lF, 1 CL s CL 3–4 CL mF	68 48 55 51 51	m m m m
					E 38 35 46 46 66	Ground mF, many CL m, few lF, 2 CL mF, CL m, lF, CL	64 103 73 68 58	m l m est
75	C 63 94 77 D 33 57 37 36	s, m, lF, CL lF mlF, CL, do, db few mlF few lF, cysts, CL mlF	160 127 141 135 160 86 134	l l l est m l	C 42 41 42 D 20 31 22 16	Frozen smF, many CL CL smF, many CL sF mF few mF	59 57 57 49 36 41 42	m m m s s s
15	C 15 25 23 29	mF mF m, lF	73 95 138 245	m est est	C 28 24 27 28	Frozen mF many sF smF	59 57 52 42	l s s
Controls	18	sF	36	s	18	sF	36	s
			B. Hypophysectomized Rats[4]					
120	A[5] 103 63 B 110 90 57 115	lF and cysts (compact granulosa, some thecal thickening); few CL; I.T. 2+	108 117 135 116 103 116	ml m l m est	B 65 56 46 57	lF, cysts (scattering and some enlargement of granulosa cells) CL of all sizes; I.T.+	114 109 107 106	m m l
90	D 52 75 68 77	as above[6] (no cysts or CL)	125 111 123 111	m est ml	D 17 44 32 36	s, smF (follicles in beginning atresia); I.T.+	65 127 95 92	m m m
75	C 40 53 48	as above	149 143 47	l	C 92 75 86	lF, cysts (scattering enlargement of granulosa cell) CL all sizes; I.T.+	93 92 90	m est
60	D 32 34 32 29	as above	108 128 107 84	ml ml m	D 30 32 29 24	sF (increased in number, but in atresia) I.T.+	105 54 76 69	ml s ml
30	B 62 24 43 42	as above	110 108 118 135	ml m est	B 49 39 56 80	lF, cysts, few solid CL; I.T.+	100 90 92 86	ml m m
15	C 21 22 23	increase sF, few smF; I.T.+	40 65 53	s s	C 20 22 24	smF I.T.+	92 86 80	m est
9	D 13 12 11	no follicular devel.; I.T.+ (partial repair)	41 32 37	s s	D 13 12 15	no follicular devel.; I.T.+ (partial repair)	31 36 32 28	sm s s
Controls	9	no foll. devel.; I.T. deficient	23	s	9	no foll. devel.; I.T. deficient	23	s

[1] 23 to 24 days at onset, one-third of the total dose implanted intramuscularly daily for 3 days with autopsies 24 hours after last implantation. For key to abbreviations see Paper I, table 2.

[2] For treatment of these glands see Footnote on table 1.

[3] The absolute potency of implants varied somewhat from one experiment to another (separated by weeks) and individual experiments made simultaneously with the same batch of glands were therefore indicated by the letters A to E.

[4] 26 to 28 days old, 6 to 8 days post-operative; implanted and autopsied as in normal rats.

[5] Experiments A to D were made simultaneously with the corresponding groups of normal rats.

[6] Picture resembling that obtained on injection of pregnant mare serum gonadotropin in hypophysectomized rats.

prostate weights, and in the case of testis weights the difference was less pronounced.

The different types of pituitaries were then implanted into immature female rats, normal as well as hypophysectomized. In normal rats fresh glands were found to produce very much heavier ovaries and uteri than frozen, stored or thoroughly ground glands whereas the minimal detectable dose as well as the incidence of corpora lutea did not appear to be different in the two groups (table 2, A).

In hypophysectomized rats both types of glands showed the same minimal dose for follicular development (15 mg.) and for repair of the interstitial tissue (9 mg.) (table 2, *B*). The ovarian and uterine weights produced by greater doses appeared somewhat higher on implantation of fresh glands. The histological examination of the ovaries, however, has revealed more striking differences. In all groups responding to fresh implants the enlarged follicles were imbedded in hypertrophic interstitial

TABLE 3. ENDOCRINE EFFECTS, OTHER THAN GONADOTROPIC, OF IMPLANTATION OF FRESH AND FROZEN SHEEP ANTERIOR PITUITARIES

			Fresh Glands					Frozen Glands				
Total dose	Rats[1]		Adrenals weight[2]	Thymus weight	Thyroid		Body wt. gain	Adrenals weight	Thymus weight	Thyroid		Body wt. gain
	Type	Number			Wt.	Histol.				Wt.	Histol.	
mg./rat			mg.	mg.	mg.		gm.	mg.	mg.	mg.		gm.
200	♂H	6	25	200	12	+	21	14	271	14	2+	17
Controls	♂H	6	10	154	7	−	2	10	154	7	−	2
90	♀H	3	23	194	12	+	15	18	210	11	2+	17
60	♀H	3	24	191	13	+	16	17	254	13	+	19
9	♀H	2	15	200	10	−	5	13	256	10	+	15
Controls	♀H	8	11	165	8	−	0	11	165	8	−	0
120	♀N	3	39	32				21	120			
90	♀N	4	38	40	14		5	25[3]	177	15		11
		4						26[4]	101	15		11
75	♀N	3	31	67				20	115			
Controls	♀N	12	18	165	15		9	18	165	15		9

[1] Responses of gonads and accessories of these rats included in tables 1, 2. H=hypophysectomized rats; N=normal rats (age, number of implantations, etc. see tables 1, 2).
[2] All adrenals of hypophysectomized rats receiving implants showed histological evidence of repair of cortex.
[3] Stored glands.
[4] Fresh, thoroughly ground glands.

tissue, the granulosa was compact and the theca thickened, solid corpora lutea appearing only rarely and at the highest level. The appearance of these ovaries resembled very strikingly that obtained on injection of pregnant mare serum gonadotropin in hypophysectomized rats. On implantation of frozen glands, on the other hand, solid corpora lutea were produced by all but the lowest levels. The enlarged follicles frequently showed early evidences of atresia. The interstitial tissue did not show the same degree of hypertrophy as it did in the other group. The appearance of these ovaries resembled those generally obtained with pituitary extracts.

When other effects were compared which were produced by implantation of fresh and frozen glands the following observations were made (table 3). Adrenal weights were considerably greater in all groups receiving fresh glands than in those receiving frozen, stored or thoroughly ground gland implants. In the 5-day test period in hypophysectomized males, fresh glands caused 4 times the effect and in the 3-day period in females double the effect at all levels as compared to frozen glands. An even greater difference was found on implantation of both types of glands into normal immature female rats. While frozen glands had only doubtful effect on adrenal weights, the same amounts of fresh gland caused a doubling of adrenal weights. In every instance

adrenal stimulation coincided with a corresponding inverse effect on the thymus. In normal female rats implantation of fresh glands led to pronounced atrophy of the thymus within 3 days while in hypophysectomized rats the growth of this gland was inhibited roughly in proportion to the observed increases in adrenal weights. The thyroid weights showed no significant differences; on histological examination, however, the thyroids of hypophysectomized rats receiving frozen glands were found slightly more stimulated than those of animals receiving the same amount of fresh glands. Similarly, regarding growth stimulating activity, frozen, stored or ground glands appeared slightly superior to fresh glands on implantation.

To study and compare the antagonistic potency of fresh, stored and frozen glands,

TABLE 4. 'ANTAGONISM' OF INTRAPERITONEAL IMPLANTS OF FRESH, FROZEN OR STORED PITUITARIES AGAINST INTRAMUSCULAR IMPLANTS OF FRESH GLANDS (NORMAL FEMALE RATS)[1]

Intramuscular		Intraperitoneal		Ovaries		Uterus		Adrenals weight	Thymus weight	Body weight gain
Total dose	Type of glands	Total dose	Type of glands	Weight	Description	Weight	Description			
mg.		mg.		mg.		mg.		mg.	mg.	gm.
90	Fresh	—		64	CL, do	134	l	51	35	4
				46 68	lF, CL	106 133	m	37 38	41 40	6 5
				83	lF, CL	162	l	30	42	7
				78	lF, CL, do	128	l	35	40	3
	—	90	Fresh	42	mlF, CL, do	153	ml	43	38	7
				40 42	mlF	90 111	m	38 41	31 39	2 5
				49	CL, do	106	ml	41	48	6
				35	lF, CL	95	l	42	40	4
90	Fresh	90	Fresh	39	mlF, CL	134	l	37	46	6
				37 50	mlF	153 142	l	48 40	35 37	1 4
				74	smF, CL	140	l	35	31	5
	—	90	Frozen	17	CL	30	s	17	80	5
				25 23	smF, CL, db	39 42	s	16 17	78 110	2 7
				25	smF, CL	56	sm	18	138	10
				23	CL	44	s	17	143	11
90	Fresh	90	Frozen	45	SmF, CL	110	l	39	62	10
				57 44	lF, cysts, CL, do	105 96	ml	45 41	25 36	4 5
				42	mlF, CL	115	l	42	24	1
				32	mlF, CL	55	sm	37	31	3
	—	90	Stored	25	CL	53	sm	24	140	8
				33 25	CL	46 51	sm	26 23	145 132	12 8
				22	CL	47	m	19	124	7
				21	CL	58	s	21	119	6
90	Fresh	90	Stored	70	m, CL, do	123	ml	37	37	6
				52 56	mlF, CL, do	132 126	l	38 41	37 35	8 7
				45	mF, CL	87	m	37	37	7
				56	lF, CL	160	l	53	30	8
Controls (12 Rats)				18	aF	36	s	18	165	9

[1] 22 to 25 days at onset, implanted daily for 3 days.

these were simultaneously implanted into the peritoneal cavity as well as by the usual intramuscular route (table 4). All three types of glands, on intraperitoneal implantation, were found to decrease to a similar degree the effect on the ovaries of fresh glands implanted simultaneously into the muscle. When, as a control experiment, the different types of glands were implanted intraperitoneally without simultaneous intramuscular implants, fresh glands produced again considerably heavier ovaries and uteri than frozen or stored glands.

Since these experiments showed the male accesssory and adrenal weight increasing effects of pituitary implants to be the ones most affected by the freezing procedure, the hormones isolated from pituitary extracts which are responsible for these effects were subjected to a similar treatment. It was found that repeated freezing and thawing did not decrease the activity of solutions of these hormones.

DISCUSSION AND CONCLUSIONS

The recorded experiments show striking differences in the response of certain organs following implantation of minced sheep pituitaries immediately after removal from the sella and only a few hours after the death of the sheep, as contrasted with glands which had either been ground thoroughly, stored for 24 hours, or frozen.

It has often been shown that factors such as solubility, rate of resorption and excretion, and others play a significant role in determining the effectiveness of a hormone. It does not appear likely that such factors may be responsible for the great differences in the effects produced by minced fresh glands on the one hand, and by similar glands after freezing or storing on the other. When fresh, stored and frozen glands were minced or ground and then extracted with physiological saline solution, the amounts of total soluble nitrogen were found slightly higher in stored, frozen or ground glands than in fresh minced tissue.

It also appears very unlikely that the observed differences were due to an inactivation of the responsible hormones in the glands during freezing or storing, since these hormones in solutions are not at all affected by such handling. It is possible that an enzymatic inactivation of these hormones is favored by the breaking up of cell walls. In that case one would have to assume that the catheptic enzymes of the pituitary show great selectivity in attacking and inactivating only certain of the various hormones secreted by the gland.

The following appears to us the most plausible explanation of the observed facts. A mechanism associated with the integrity of the cell may be necessary for the production of the striking effects of fresh implants, possibly active secretion or liberation of the respective hormones by surviving cells. On the basis of this working hypothesis it becomes clear why stored, frozen and thoroughly ground tissue give poor effects, in certain respects, as compared with fresh minced tissue.[2]

It has already been reported in Paper I that all extracts of sheep pituitaries are very ineffective in increasing female ovarian and male accessory weights; similarly very great amounts of such extracts are needed to produce effects on the adrenals of our rat colony. It would appear, therefore, that the content of the pituitary gland is low in the hormones responsible for these effects. On the other hand, the striking stimulation of these organs observed on implantation of small amounts of fresh pituitary tissue indicates that great amounts of these hormones can be secreted under comparatively unfavorable conditions by surviving cells.

To arrive at quantitative conclusions regarding the gonadotropic effectiveness of different types of implants, seminal vesicle weight increases listed in table 1 (also table 1 in Paper I) and other data have been employed to plot dose response curves for the two types of implants, as well as for crude pituitary extracts. These curves have been compared with similar curves for a male urine gonadotropin (prospermin, Squibb) as well as with those obtained from standardizations by Dr. Fevold for our crude pituitary extracts and pure ICSH (see Paper I, table 1). It was found that approximately 16 mg. of fresh pituitary tissue corresponded to 1 unit (100% increase in seminal vesicle weights) while about 800 mg. (or 50-fold) of frozen glands were needed to obtain this effect either on implantation or in the form of extracts.[3] When

[2] The ineffectiveness of implants of bovine pituitary tissue 8 hours old as contrasted with 1-hour-old tissue has already been recognized by P. E. Smith and E. T. Engle (1). Decreased potency of implants after storing and freezing was also recorded by A. Wallen-Lawrence and H. B. van Dyke (2).

[3] Owing to the difficulty of administering sufficient amounts or equivalents of frozen or stored pituitary tissue, to get significant responses (over 100%), the determination of the dose corresponding to a unit as well as the slope of the curve was in this case based on only 2 groups of rats: 1 group receiving 1 gm. in the form of frozen implants and one receiving an equivalent of 2.5 gm. in the form of an alkaline extract of acetone dried pituitaries (table 1, 9: also Paper I, table 1).

assayed by Dr. Fevold, only one-tenth of this amount (80 mg. equivalent) of a similar extract of our glands were needed to cause 100% increase in seminal vesicle weights. The curves obtained on plotting units against responses were the same for all these diverse preparations including that for the urinary product, about 3-fold of the unit giving 200% increase in seminal vesicle weights in each case (fig. 1). These curves were also identical with those obtained for pregnancy urine and pregnant mare serum gonadotropin in normal and hypophysectomized rats. In view of the great differences in the FSH content of some of these preparations, it appears that in the Long-Evans strain of rats FSH has no 'augmenting' effect on the male accessory

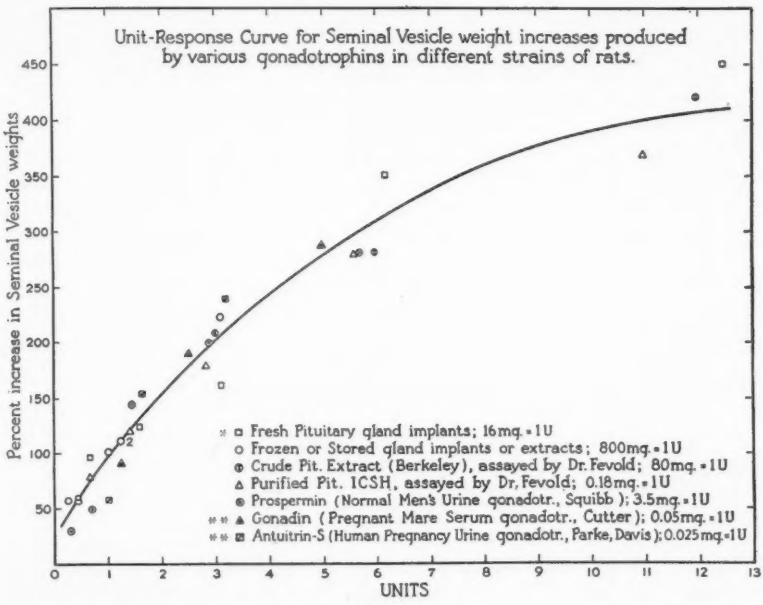

FIG. 1

* Hypophysectomized rats were used for this assay. In all other experiments listed on this figure, normal rats were used.

** The responses of hypophysectomized rats to these preparations were similar, though slightly higher.

stimulating power of the other hormone during a 6-day assay period (as has already been shown in Paper I, table 4).

While in the male rat fresh implants were found 50 times more potent than frozen implants and extracts, the differences in the female appear of a more qualitative nature. Thus in normal immature females the minimal effective dose is the same in both groups (15 mg.); while higher doses produce very much heavier ovaries and uteri in the group receiving fresh implants than in that receiving frozen or stored implants. The qualitative difference in the appearance of the ovaries in hypophysectomized rats on injection of both types of glands has already been mentioned. Also in these rats the minimal dose for follicular stimulation and interstitial tissue repair was found to be essentially the same for both types of glands, so that the observed differences in other respects could not be due to a change in the balance of the two known hormones, FSH and ICSH.

These facts seem to point toward the existence of a gonadotropin in fresh but not in frozen implants which resembles Fevold's 'LH' in that it causes striking weight increases of seminal vesicles in male rats and of ovaries in females. It has been demonstrated in Paper I that these are not properties of the ICSH, while they characterize certain urinary gonadotropins. Thus it would seem as if the qualitative differences observed between fresh and frozen implants could be explained only by the assumption of the existence of 2 active substances. Evidence presented in Paper I, however, has indicated that one substance may give pure ICSH effects in one strain of rats while giving the effects typical for 'LH' in another. The assumption that ICSH in pituitary extracts might represent the precursor for the active hormone was suggested as an explanation for these phenomena. The differences observed upon implantation of fresh as contrasted with frozen, ground or stored tissue which have now been reported, seem to represent additional evidence favoring this assumption. ICSH with its masked properties would then be present only in the pituitary and in extracts of this gland, whereas living or surviving tissue would convert it into the free hormone upon secretion, a conversion which in certain rat strains may also be possible outside of the pituitary. The freed hormone, characterized by giving 'LH' effects also in the Long-Evans strain of rats, would occur only in the serum and urine of normal, nonpregnant human beings and animals.

The recorded experiments appear to indicate that also adrenocorticotropic hormone is actively secreted by fresh implanted pituitary tissue, while being present in only small amounts in frozen implants and extracts. No indications have been obtained whether, in case of this hormone, we are dealing with qualitative or only quantitative differences.

The possible slight inferiority of fresh glands in regard to growth and thyrotropic potency may be due to antagonizing effects exerted by the sterol hormones produced at a high rate by the adrenals and the gonads of the recipients of fresh implants. It appears most likely that this mechanism is responsible for the observed inverse relationship between adrenal and thymus weights in all groups (3), if it is kept in mind that the thymus weight increasing effect, associated with the action of growth hormone in hypophysectomized rats, is present to a similar extent in both groups of implanted animals.

In general it appears from these studies, that frozen glands should be preferably implanted if an investigator wishes to determine the hormonal content of an endocrine gland, while the implantation of fresh glands will give him valuable indications of the potential availability of hormones, i.e., of the amount of hormone that can be liberated by the gland in heterologous surroundings and without nervous or circulatory connections. Attempts have not yet been successful to reproduce the conditions of implantation in vitro and thereby to cause great amounts of 'LH' and adrenocorticotropic hormone to be 'secreted,' into the surrounding liquid. When this problem will have been solved, chemical isolation and purification of the respective hormones will be greatly facilitated.

SUMMARY

Evidence has been adduced that there are pronounced differences regarding certain effects of daily implantation of fresh minced as contrasted with frozen, stored or thoroughly ground sheep pituitaries into immature normal and hypophysectomized rats.

In regard to their gonadotropic effects, small amounts of fresh glands produced great increases in the weights of the accessories of normal and hypophysectomized males and similarly pronounced increases of ovarian weights in females. Frozen and stored glands were found very much inferior in both respects; although the minimal

effective dose for FSH and ICSH, as determined in female hypophysectomized rats, was found to be similar in both groups.

In regard to adrenocorticotropic effects, fresh implants were also found to be considerably more active than extracts and stored or frozen gland implants.

Fresh glands after thorough grinding proved very much less effective than minced tissue and slightly more effective than frozen or stored glands.

No differences were observed in growth stimulating or thyrotropic potency of the two types of implants.

The assumption that fresh minced tissue might be able to 'secrete' more hormone than can be extracted from 'dead' tissue appears to afford the best explanation for the quantitative differences observed between fresh implants and frozen implants or extracts. Qualitative differences observed in the case of the gonadotropins were discussed in the light of a previously proposed working hypothesis; they were interpreted as evidence favoring this hypothesis.

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HEMOCONCENTRATION ACCOMPANIED BY A LOW SERUM CALCIUM IN VAGOTOMIZED, PARATHYROIDECTOMIZED DOGS[1]

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THAT PREVIOUS vagal section will greatly attenuate parathyroprival tetany in the dog has recently been demonstrated in this laboratory (1). The few deaths which did occur were of interest in that the laboratory data indicated that relative hemoconcentration (increased proteins and hematocrits) might be a contributing factor in the so-called depressive death in parathyroprival tetany. It was thought that a more intensive study of this hemoconcentration should be made to determine if it is accompanied by any change in the serum potassium or sodium, such as is known to accompany adrenal cortical insufficiency.

METHODS OF ANALYSIS

The serum calcium was determined by the Collip and Clark modification of the Kramer and Tisdall method except that a flusher was used and the precipitated Ca oxalate washed only once. This method was found to be accurate to within 1%. The serum potassium was determined by the Consolazio and Talbott (2) modification of the chloroplatinate method, and we can confirm their findings that this method is accurate to within 2%. Inorganic phosphorus was determined by the Briggs modification of the Bell-Doisy procedure. Serum sodium was determined by the Hoffmann and Osgood micromethod (3). The serum proteins were determined by gravimetric weighing after acetone precipitation. Hematocrits were taken on all samples, using heparin as the anticoagulant. The dogs were kept on a constant diet of commercial dog biscuits and water. The blood and serum specific gravity were determined by the falling drop method.

EXPERIMENTAL

All of the dogs studied had their vagi severed above the diaphragm by a thoracic operative approach. This was done 4 weeks or more before the total thyroidectomy. The thyroid tissue was excised widely in order to remove as much as possible of the parathyroid tissue. Control samples were obtained before the operation and every 2 to 4 days postoperatively. Both sexes of dogs of all sizes were used. One very old dog (teeth worn down) and several puppies were used in order to ascertain the effect of age on the depressive death syndrome.

[1] Read at the twenty-fourth annual meeting of the Association for the Study of Internal Secretions, New York City, June 1940.

RESULTS

Figure 1 records the effect regarding Ca and hemoconcentration of total thyroid, ectomy in an old dog. With the exception of the protein and K levels, the 2 control samples show minor changes consistent with the hematocrit and blood specific gravity changes. The serum Ca did not decrease below 5.3 mg.%, and the protein and hematocrit indicate that only an initial hemodilution recurred with the protein level returning almost to the initial level at the end of 4 weeks. With the depression of the serum Ca the K decreased from 5.2 to 3.6 m.eq./1. The hematocrit indicates the anemia which the surviving animals develop after total thyroidectomy. This dog, aside from lessened activity, was entirely symptomless in spite of the marked reduction of the serum Ca level.

Figure 2 records the effect of total thyroidectomy in a growing dog less than 8 months old. Five days after operation this dog showed beginning depression and

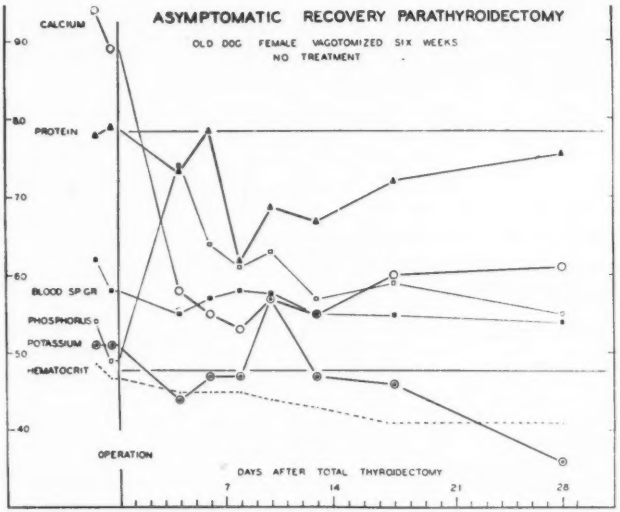

Fig. 1.

muscular rigidity. With the decrease in the level of serum Ca to 6.2 mg.% on the 5th day and 4.5 mg.% on the 7th day, the serum protein rose progressively. The injection of 150 cc. of 0.9% NaCl solution resulted in a slight decrease in the protein and hematocrit levels. Whereas the hemoconcentration did not respond to a small intravenous dose of Ca lactate (10 cc. of 2%), a hemodilution did result from the oral administration of Ca lactate. On the 12th postoperative day the oral Ca was discontinued, and the depression, tremor, rigidity and asthmatic wheezing with cyanosis grew progressively worse until the 16th day. At this time the dog did not respond to intravenous Ca therapy, and a blood sample taken before death clotted normally, but the expressed serum "jelled" while being centrifuged. This prevented blood chemistry and protein determinations. The hematocrit and specific gravity, however, give evidence of increased hemoconcentration. The serum K sharply decreased on the 7th postoperative day and remained at 3.6 m.eq./1. The serum Na was within the normal range at the time of maximum hemoconcentration. In this

young animal the serum P rose sharply to 10.7 mg.% on the 5th postoperative day and then showed synchronous fluctuation with the Ca levels. These initial studies indicated that young, full grown dogs would perhaps produce the desired moderate symptoms of the depressive syndrome.

Figure 3 represents a moderate depressive syndrome which, aside from spontaneous decrease in activity on the part of the dog, was entirely asymptomatic. An initial hemodilution occurred, as is reflected in the lowered serum proteins and hematocrit levels. Figure 4 represents a similar type of response in an untreated female dog. The two original control samples agree adequately. The serum protein, and to a lesser extent the hematocrit, are in inverse correlation with the fluctuations in the serum Ca level. The serum K and P tend to fluctuate in proportion to the Ca. The

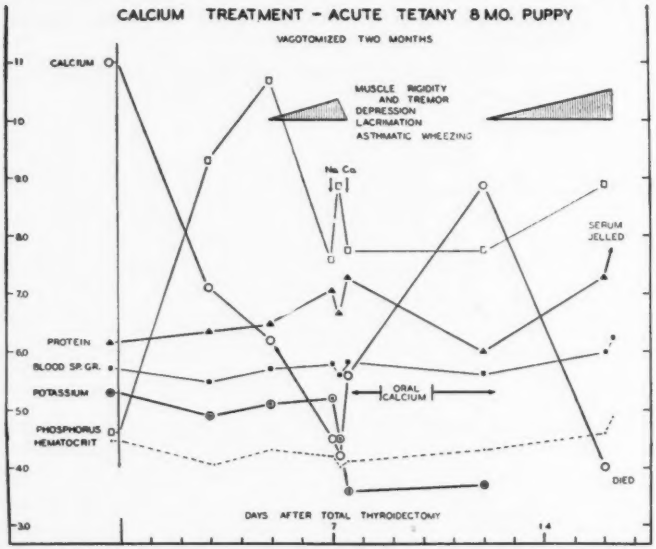

Fig. 2.

fluctuation of the specific gravity indicates hemoconcentration with a decreased Ca level. The serum Na at the times of the greatest hemoconcentration did not differ from the original control level. Figure 5 represents the data on a young male dog which was vagotomized above the diaphragm as a puppy to determine the possible effect on growth. (There was no difference in growth of this dog compared to a litter mate of the same sex). The serum proteins and hematocrit indicate a hemodilution for the first 2 weeks after parathyroidectomy. As the serum Ca fell below 5 mg.%, the dog began to show symptoms of depression, and relative hemoconcentration obtained. This responded only slightly to 0.9% NaCl, while the symptoms of gastro-intestinal stasis, depression, slight tremor, asthmatic wheezing with cyanosis, sneezing, lacrimation, constant whimpering, and rubbing of the head with the forepaws persisted. When Ca lactate, 0.5% in 200 cc. Ringer's solution, was given, the dog vomited some putrid food which had been ingested 48 hours previously, and the symptoms disappeared only to return 2 days later. Another injection of 200 cc. of Ringer's plus Ca lactate was given, and the protein and hematocrit showed a fairly marked

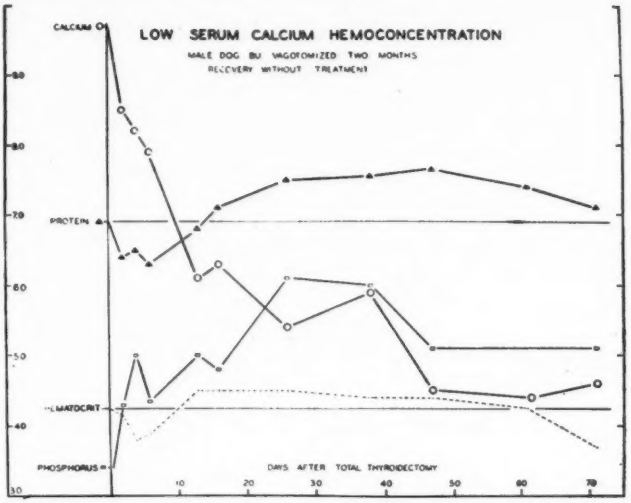

Fig. 3.

drop with complete relief of the symptoms, which did not return. The serum K expressed in m.eq./l., was within normal limits throughout. The hematocrit shows the constant downward trend characteristic of the anemia which these dogs develop. Figure 6 represents the development of a similar hemoconcentration accompanied by

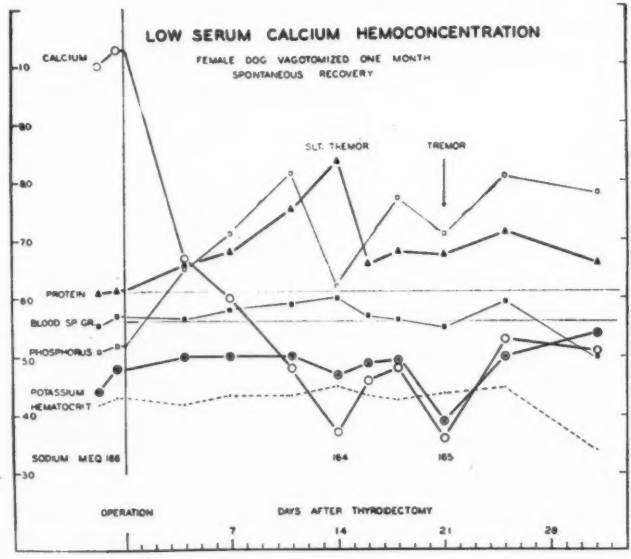

Fig. 4.

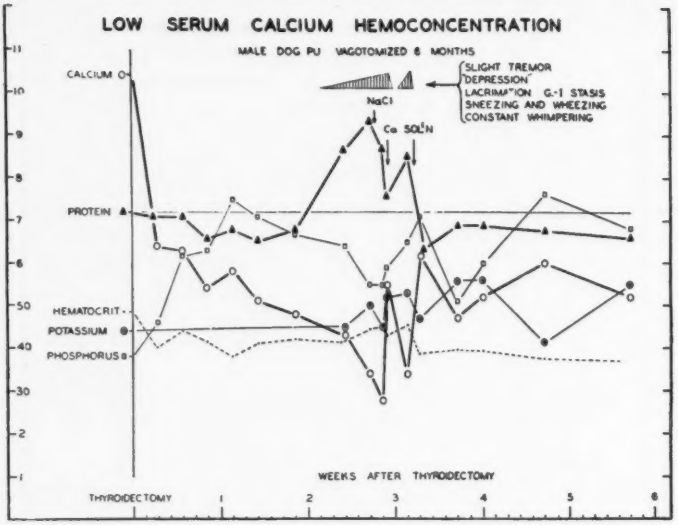

Fig. 5.

a normal Na and a lowered K when the serum Ca falls below 5.0 mg.%. A hemodilution obtained for the first 11 postoperative days. The dog developed depression, lacrimation, rigidity and slight tremor on the 12th day. This was allowed to progress until the 15th day when the dog was given 0.5% Ca lactate in Ringer's. When no

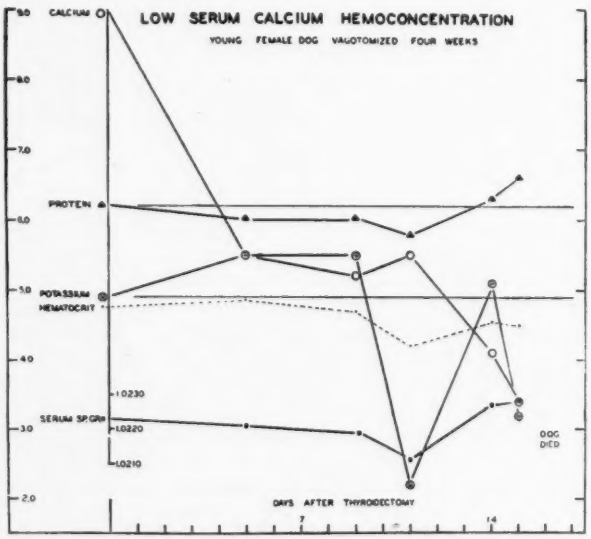

Fig. 6.

response was obtained, an injection of adrenal cortical hormone (10 cc. Upjohn) was given intravenously along with 200 cc. of Ca lactate and Ringer's solution. The dog responded to this additional treatment, but relapsed after 24 hours and died.

DISCUSSION

Due to the development of anemia in these dogs, the hematocrit is not a good index of hemoconcentration unless a line drawn between the control and the final reading is used as the base line. The cause for this anemia is as yet undetermined. Two possible explanations should be considered. Thyroidectomy in the rabbit has been shown to produce an anemia by Kunde and her coworkers (4), and the lack of P absorption from the gut, due to a high serum P, may inhibit the absorption of iron, since Elvehjem and his coworkers (5) have recently shown that iron is absorbed with P.

One of the symptoms not noted in the illustrations was that of intense diuresis with the decrease in Ca and the rise in serum proteins and hematocrit. At the time of the hemoconcentration the urine flow (as noted in bladder fistulae dogs) amounted to only 1 or 2 cc. of blood tinged urine per hour. The allergic symptoms, namely sneezing, lacrimation and asthmatic wheezing, need emphasis in that these dogs demonstrate that some other cause than true allergy may produce these symptoms. Other dogs show evidence of constant pain by whimpering, and at the same time they paw at their heads or rub their heads against the side of the cage. This, accompanied by squinting and lacrimation, frequently gives the impression that they may have headache.

Because these data are not consistent with the classical findings of adrenal cortical insufficiency, such a possibility may be excluded. Since approximately 50% of the serum Ca is bound to a protein fraction, and since the observed rise in protein is found to occur when Ca drops below a serum level of 5.0 mg.%, one might wonder if this rise in protein is compensatory in an attempt to substitute protein-bound Ca for ionized Ca. Since McLean and his coworkers (6) have shown that even at low levels the ratio of bound to unbound Ca remains essentially the same, and since the hematocrit usually rises with the protein, this protein rise most probably reflects only hemoconcentration.

It has been previously observed in bladder fistulae dogs in this laboratory (7) that when the blood level of Ca or K alone was given orally in 0.45% NaCl a diuresis above the control level occurred. When the two "diuretic ions" were given together, no diuresis above the control curve occurred, and an actual retention of chlorides obtained. These studies have since been elaborated and fortified by fluid balance studies on Ca and K in rats (8). The more recent rat studies indicate that the maximum retention of these two ions occurs at a proportion of 3 K to 1 Ca, which is approximately the ratio of the ionized levels of the blood serum. Various suggestive studies in the literature indicate that there may be a complex carrier for these two ions across the renal tubules. Preliminary studies in this laboratory indicate that this carrier involves glucose. If such a carrier is formed only in the presence of a definite ratio of Ca to K, and if Ca and K tend to maintain the blood volume, then 4 types of hemoconcentration should be theoretically possible, namely: (*a*) high serum Ca, (*b*) low serum Ca, (*c*) high serum K and (*d*) low serum K hemoconcentration.

High serum Ca hemoconcentration after excess parathyroid administration has been studied by several workers (9–13), and it can be ascribed to excess Ca ion, since it also follows overdosage of vitamin D or Ca salts. The data of this study tend to establish low serum Ca hemoconcentration as a definite syndrome, since the serum K, instead of being elevated, tends to be reduced with the fall in Ca. High serum K

hemoconcentration is well established clinically and experimentally where it is known to accompany adrenal cortical deficiency. A survey of the literature does not reveal any studies in low serum K hemoconcentration, but this may be due to failure to observe it, or it may be masked by more serious symptoms such as paralysis (14). Suggestive of this hemoconcentration is Heppel's (15) observation of a marked decrease in the serum chlorides of rats on a K free diet which lowered the serum K to 2.0 to 3.0 m.eq./1. More pertinent study is necessary on this theoretical phase.

SUMMARY

Parathyroidectomy in dogs vagotomized above the diaphragm frequently leads to a low serum calcium hemoconcentration, as judged by a rise in the serum proteins and the hematocrit. The serum potassium tends to decrease with the decrease in serum calcium, while the serum sodium levels remain within the normal range.

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DOES TESTOSTERONE PROPIONATE INHIBIT OVULATION?[1]

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IN A RECENT REPORT (1) on the effects of testosterone propionate on the corpora lutea of early pregnancy in mice, it was shown that the ovaries were stimulated by small dosages but depressed by large amounts of this androgen. Just how much the ovaries were stimulated was not determined but in the course of those autopsies, and several times since, we have observed ovulations which had occurred during the series of injections. Some typical examples follow.

Mouse 42 had been injected daily since mating with 0.5 mg. testosterone propionate and was autopsied on the sixth day after the vaginal plug. On the right ovary were 2 corpora albicantia and 4 good corpora lutea. Two blastocysts were recovered from the uterine flush (from the ovulation at the time of mating) and 4 one-celled ova were found in the ovarian third of the tube. Two of these one-celled ova were still surrounded by granulosus cells. This second ovulation probably took place late on the fifth day or on the sixth day after the vaginal plug. The right tract had 2 corpora lutea and 2 unfertilized ova were recovered from the ampulla.

Mouse 44 had been treated the same as *mouse 42* except that the injections went 7 days and the animal was autopsied on the eighth day. On the left ovary were 4 small corpora albicantia and 1 corpus luteum. One degenerate egg with intact zona was found in the lower portion of the tube. From the appearance and the position of the egg, ovulation had taken place about 2 days earlier.

DISCUSSION

The literature on the effects of testosterone propionate in the female animal is still in a state of confusion because of the various ages and kinds of animals experimented upon as well as the amount and number of injections. Starkey and Leathem (2) Salmon (3, 4), Nathanson, Franseen and Sweeney (5) are among those who report that this androgen has a gonadotropic effect on the follicles while Korenchevsky, Dennison and Hall (6), Salmon (3, 4), Nathanson, Franseen and Sweeney (5), Hohlweg (7) and Freed, Greenhill and Soskin (8) all show that corpora lutea are present in the immature rat 96 hours or more after a single injection of 1 to 5 mg. testosterone propionate. On the other hand, Boling and Hamilton (9) report that the later stages of follicular growth, maturation, and ovulation are suppressed in the guinea pig.

Therefore, several questions arise from these observations. Does testosterone propionate stimulate the anterior pituitary to secrete sufficient gonadotropic substance to cause ovulation or do these ovulations occur in cyclic order in spite of the injections? Are the corpora lutea reported by those above the result of an ovulation which occurred during the course of injections or is there evidence that these corpora develop because of excessive luteal stimulation without ovulation?

[1] The testosterone propionate was furnished through the courtesy of Doctors Gregory Stragnell and Max Gilbert of the Schering Corporation and Dr. R. Macbrayer of Ciba Pharmaceutical Products, Inc.

SUMMARY

In a series of mice receiving daily injections of 0.5 mg. testosterone propionate several animals were seen which had ovulated during the course of injections. It is not known whether these ovulations occurred because of, or in spite of, the injections.

Several pertinent questions are raised and it is hoped that other investigators will more closely watch for this phenomenon.

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THE EFFECT OF THYROXIN ON THE SOCIAL ORDER IN FLOCKS OF HENS[1]

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THE PRESENT REPORT is one of a series which deals with the physiological analysis of the peck order in flocks of hens by means of treatment with various hormones as suggested by Allee (14). Detailed reports have appeared on the effects produced by injections of testosterone propionate (15) and of estradiol (16) and there has been a preliminary report of the lack of effectiveness of epinephrine in this connection (17). The description of our work with testosterone (15) may be consulted for many of the details of care of the birds and general procedures of observation which will not be repeated here.

The social order in flocks of hens is based primarily on initial encounters between individuals during which the dominance relations are settled either by fighting or by passive submission of one member of the contract pair (1–13). The winner usually continues to exercise the peck-right so won at frequent intervals in the daily life of the flock. Once firmly established, the social order is fairly stable among hens.

Basis for Study

Doses of thyroxin, sufficient to induce moulting out of season (18) and so produce a drain on the bird's energy might be expected to decrease aggressiveness below that of untreated flock mates. There are other possible effects, since at the height of the normal or of an induced moult, egg laying usually ceases, the comb shrinks presumably because of a decreased supply of androgens in these endocrine hermaphrodites, and weight decreases. Normal changes are not so severe as those produced by our strongest dosage.

Treatment with thyroxin in too small amounts to induce moulting and loss of weight, or at least too small to have much effect on birds in these respects, might conceivably reduce social effectiveness. This would, perhaps, be expected from the results obtained with higher dosage, particularly since there is evidence that such artificial hyperthyroidism results in a loss of androgens by males (19, 20) and we have demonstrated (15) that an increase in testosterone causes hens to move up in the social order of their flock.

On the other hand, the injection of smaller doses of the thyroxin might increase aggressiveness. This would be in line with Winchester's (21) finding that injection of thyroxin counteracted the decrease in egg laying which otherwise followed thyroidectomy in White Leghorn hens. Increase in egg laying is correlated with an increase in comb size (22), and size of comb is mainly determined by the amount of androgen present.

[1] This work was supported in part by a grant from the Committee on Research in Endocrinology of the National Research Council. We are indebted to Dr. L. V. Domm for his careful and critical reading of this paper in manuscript and for preparing certain poulards.

The dividing line between the opposing effects of hypo- and hyperthyroidism might coincide with the existing level of thyroid secretion in a so-called normal fowl, or it might lie above or below that level. If the latter, then a slight increase in thyroxin could initiate a rise in social status through its effect on the gonads. Further, certainly with mammals and apparently also with birds (21, 23), thyroid medication increases the metabolic rate and, for all we know to the contrary, an increase in metabolic rate may increase the type of social effectiveness which we have been measuring. An apparent confirmation of the reasoning that a proper dosage of thyroxin might cause hens to become more aggressive appeared promptly in the report (24) that thyroid extract administered to the lizard, *Anolis carolinensis*, increased its "pugnacity" as indicated by territorial reactions.

MATERIALS AND METHODS

Only White Leghorn hens were used in our experiments. They were housed, with one exception, in 4 similar pens each of which had about 70 sq. ft. of floor space. The pens and treatment have been described in some detail elsewhere (15). C. was in direct charge of the birds and with the aid of B. made the primary observations. There was no other caretaker.

In addition to incidental records made during other work with the hens, careful observations of social reactions lasted from 3 to 5 hours a day for 5 to 7 days a week. Pair contact reactions (pecks) were recorded only when there was active or threatened pecking and one of the pair obviously retreated.

Five flocks were used in investigating the effects of thyroxin from late March to early August and the hens were watched daily for after effects until the end of August, 1939.

Flock A 6 consisted of 3 birds. One of these died of leucosis with which the birds were infected when first received by us from Iowa State College. The other 2 were older hens which came originally from *Flock D*. Unlike any of the other flocks, these birds were housed in a neighboring greenhouse. Their pen had about the same floor space as the others but was otherwise only roughly comparable.

Flock B 1, 2 included 9 highly inbred young hens (25), from Iowa State College. They were somewhat over a year old and included 3 poulards; one of these was partially masculinized. Three of this flock, including the masculinized poulard, died during the summer. One of the hens was also treated with testosterone propionate.

Flock C was composed of random-bred pullets which had been battery reared (26) in our laboratory from newly hatched chicks received late in September 1938. *RY* and *BB* died during the course of the experiments; both had received thyroxin.

Flock D was made up of 9 old hens from an Indiana stock that had been line bred for 18 years. They were received on October 22, 1937. *RR* and *RG* died as a result of treatment with thyroxin.

Flock E included 8 culled hens from an Indiana Farm Bureau strain that had been line bred since 1933. The birds were received in mid-April, 1939, when they were about one year old. Five of this flock died during the experimental period; one, as a result of extreme heat. *GG* died while she was being injected with thyroxin and *BB* died 15 days after her last injection.

All of these flocks with the exception of *E* had been experimented with previously, using hormones other than thyroxin.

The hens injected with thyroxin, the doses used, the periods of treatment, weight, social status and other pertinent data are summarized in table 1. Synthetic crystalline thyroxin (Roche-Organon) was used throughout. It was dissolved in alkaline solution at a concentration of 5 mg. per cc. Controls received the same or

TABLE 1. DETAILS OF TREATMENT WITH THYROXIN[1]

Series	Duration	Flock	Individual	Weight, oz.	Dose, mg.	Social rank	No. of doses	Days from 1st to last dose inclusive	Remarks
				Spring injections					
1	3/30 to 4/19	D	RG	64.0	7.00	I	5	9	Died under treatment
			RR	57.5	5.00	V	6	11	Injected every other day
			RW	65.0	6.00	Last	6	11	
			♀♀	53.0	7.00	Last	1	1	Received one dose only
2	4/22 to 6/15	D	GG	64.5	4.00	I	3	9	
			BW	63.0	3.00	I	11	41	
3	4/22 to 6/15	C	R♀	60.5	0.50	IV	9	33	
			PR	57.5	0.25	V	9	33	1 mg., first day only
			GG	52.5	0.25	Last	4	13	
		D	R♀	68.5	0.75	V	8	29	
			BR	67.5	0.75	VII	4	13	
			RW	59.0	0.75	Last	4	13	
			♀♀	59.0	0.75	Last	4	13	
		E	GG	81.5	0.75	IV	12	45	2 mg., first day only; died under treatment
			BB	71.0	0.50	V	9	33	
			BR	62.5	0.25	Last	4	13	
				Summer injections					
2S	6/6 to 8/2	B 1, 2	RG	54.5	1.5–1.0	Last	8	29	4 of each strength
		D	BR	63.0	1.5	V	9	33	2 mg., first day only
		E	PP	86.5	4.0–3.0	II	2+4	29	7/5–7/9; 7/13–7/17; 7/29–8/2
3S	6/6 to 8/2	A 6	G♀	66.5	2.00	I	10	37	
		B 1, 2	♀♀	52.5	3.00	I	4	13[2]	2 mg. first day only
			BW	62.5	3.00	II	4	13	2 mg. first day only
			B♀	48.0	3.00	III	3	9	Broody
		C	BB	60.0	1.50	III	7	25	Died under treatment
			R♀	59.0	1.25	IV	10	37	
			GG	53.5	1.00	Last	12	45	
		E	BB	60.5	0.75	V	4	13	Died 15 days after last injection
			BR	63.5	0.75	Last	4	13	

[1] All except *series 1* were injected every fourth day.
[2] 1.25 mg. testosterone daily.

nearly the same volume of 0.8% NaCl solution made slightly alkaline. Injections were made subcutaneously in the side of the breast, never more frequently than every other day and, with the exception of the first 4 hens treated, no oftener than every fourth day.

Influence of Thyroxin on the Social Order

Series 1. ♀♀ was given one injection only of 7 mg. on March 30 and lost 5 ounces in weight; the others lost 18 to 22 ounces by the fourth day after the last injection. All were thrown into a severe moult. There was a cessation of egg laying, a shrinking and drying of the comb to a capon-like condition, a loss of appetite, a shrillness of voice, general inactivity and a dejected appearance. As with weight, ♀♀ showed most of these effects to a lesser degree and for a much shorter period.

RG and *RR*, which occupied high and middle ranks respectively, lost their peck-rights over all of their untreated subordinates. The low ranking *RW* was

pecked 3 times by *YY*, her only subordinate; *YY* then retreated when threatened by *RW* and the same reaction was seen twice on the following day. *RW* was placed in isolation because of her severe moult before other contacts between the 2 hens was observed. *YY* had only one subordinate, *BR*, and managed to hold the latter in subjection. She also won over *RR* and *RG* when they were strongly affected by their treatment with thyroxin. Pronounced effects continued long after the injections were stopped. The treated birds practically ceased pecking their underlings and spent most of their time standing quietly and drooping in a dejected manner as sick birds generally do.

RG, which received the most thyroxin given any of our hens, developed polydipsia and diarrhea and finally became too weak to fly to the roost at night and required forcible feeding. When these experimental birds became too helpless to run and hide from their flock-mates, which persecuted them with increasing intensity and finally killed *RR*, the other 2, *RG* and *RW*, were isolated. *RW* recovered and laid an egg on May 11, on which date she was permanently restored to *Flock D*, where she was dominated by all. *RG* succumbed.

No reversals which involved only normal controls took place during this period of observation, or for that matter, during the entire time of our observations now being reported. A week before, *RY* of *Flock D* won the peck right over her dominant, *BY*, which at that time was having considerable difficulty from a respiratory ailment.

Series 2. The hens in this series were given 3 to 4 mg. every fourth day for longer or shorter periods in late spring. This treatment induced a much less severe moult than was given by the birds in *series 1*; nevertheless they moulted more or less completely. Other effects were as follows. The hens lost from 8 to 15 ounces in weight by the fourth day after the last injection. Egg laying decreased decidedly but did not cease. The comb increased in size as much as 17 mm. (length plus height) but decreased as much or more within 16 days following the last injection; the decline tended to coincide with the increasing severity of the moult. General activity was only slightly or not noticeably depressed. The control hens in the flock either showed none or little of these changes.

Series 3. These 10 birds received 0.25 to 2.0 mg. of thyroxin no oftener than every fourth day. This dosage produced only slight moulting if any. Some hens did not lose weight, while others lost up to 12 ounces by the fourth day following the last injection. There was no definite effect on laying, which showed much variation both in controls and experimentals. The combs tended to increase in size somewhat but not significantly more than did those of the controls. Appetite and activity were not noticeably affected.

All the hens in *series 2* and 3 maintained their social positions unchanged.

During June and July, certain hens selected from various social levels were injected with thyroxin with results which are roughly comparable with those obtained in *series 2* and 3 during the spring. These summer series are designated as *2S* and *3S*; the classification is made on the basis of physiological reaction of the birds rather than on dosage. When individuals which had received small amounts of thyroxin in the spring were included they were given slightly more thyroxin than before, in an attempt to increase the effectiveness of the treatment without throwing the birds into a definite moult.

The birds in *series 2S* were somewhat more affected by the treatment than were the hens in *series 2*. With these hens, moulting varied from slight to pronounced; weight dropped from 9.5 to 27.5 ounces; egg laying stopped (however, *PP* had never laid); and the comb decreased in size 23 to 26 mm. *GG* of *Flock C* proved to be fairly sensitive to the 1.0 mg. dose which she received and may, perhaps, be classified

with this group, although decided effects of thyroxin were delayed. GY of *Flock A6* developed a state intermediate between the arbitrary groups 2S and 3S. A definite but incomplete moult was induced; she lost 5 ounces more than her control; her comb was relatively unchanged and she continued to lay. GY's general behavior appeared in no way abnormal.

There were no changes in social level of either the treated birds or controls during the summer, excluding changes resulting from illness and death.

Influence of Thyroxin on Initial Encounters

Once the social position of a hen in her flock has been decided, she tends to retain that place. Since social status in flocks of hens is originally determined largely on the results of initial encounters, at least with birds that are not reared together, it is possible to secure a more sensitive indication of the combination of aggressiveness and fighting or bluffing ability that determines their rank by staging pair contacts between birds from different flocks than by waiting for the rare changes in the established order.

The general technic used in making such observations has been described elsewhere (15). These encounters should be held in pens strange to both members of the pair. Both birds should be introduced simultaneously and watched until the peck right has been established either by combat or by passive submission. As a partial control over fatigue no bird should be matched with another more than once a day.

Two series of such encounters were conducted, one during the spring and another in the summer of 1939. The relation of the results of these encounters to thyroxin injections, when analyzed, showed that in cases where a bird given a relatively high, but not a pathological, dose of thyroxin (*series 2* or *2S*) met a strange hen, the treated individuals won 50% of their 38 contacts. Their untreated flock mates won 53% of 230 similar contacts with untreated strangers. Hens given lower doses of thyroxin (*series 3* or *3S*) won 54% of their 117 initial encounters. These differences are not significant.

There are, however, other factors to be considered. Three of the five hens in *series 2* and *2S* ranked high in their local flocks and the hens in *series 3S* which received the larger doses also ranked high socially. These hens were naturally strong and aggressive, and, other things being equal, might be expected to win a relatively higher percentage of their bouts than the controls. For this reason the results were recalculated, taking into account the different factors such as position in the social order, size of comb, condition as regards moulting and weight, which previous work had indicated could be used to indicate the probable outcome of staged initial encounters.

When allowance was made for these presumed advantages, the hens treated with thyroxin in fairly large doses, physiologically considered (*series 2* and *2S*), won 68% of the 25 contacts they were expected to win. Meantime their controls won 84% of the 9.5 contests in which they were predicted to have an advantage over these *series 2* and *2S*, thyroxin treated hens. The lower doses of thyroxin (*series 3* and *3S*) had practically no effect on the predicted outcome and the results differed little from those given by controls.

The records of the hens under thyroxin treatment when compared with those made by the same individuals when they were not being treated showed this relationship somewhat more clearly. Hens in *series 2* and *2S* were decidedly less aggressive when under the influence of thyroxin. Those in *series 3* and *3S* did not exhibit any detectible difference in aggressiveness, whether treated or not.

Our data indicate then that pathological doses of thyroxin produce so marked

a reduction in social successes that changes occur in the social rank of the home flock; high, but not pathological amounts, produce tendencies in the same direction but not enough to affect ranking in flocks, where social inertia helps hold the peck order stable. Smaller injections have no social effect on behavior which can be measured by the methods which we have employed.

Other Effects of Thyroxin

The pronounced influence of experimental hyperthyroidism on moulting, body weight, size of comb and egg laying is already established. The first two were found as early as 1912 (27). These results have been confirmed and extended by others particularly by Zawadowsky and his associates (20, 28, 29, 30). Our experiences add little; we observed such reactions in part as a rough assay of the physiological potency of the thyroxin dosage on different individuals. As a rule no moulting occurred when less than 1 mg. of thyroxin was administered every 4 days. The degree of delay before the onset of moult was roughly proportional to dosage.

With two exceptions, the hens were not moulting when thyroxin treatment began. *RW* of *Flock D* in the summer, was just completing an induced moult from heavier spring injections and *BR* of *Flock E* had lost a few feathers and was judged to be in stage 1 of moulting, if the entire range of moulting intensity is considered in terms of 5 arbitrary stages based on severity of the moult. Some of the controls began moulting without treatment during the period covered by the experiments.

Decrease in weight occurred among the treated hens as was to be expected and provided a convenient, if rough, assay of the effectiveness of the dosage for different individuals. Birds that received less than 1 mg. every 4 days, with the exception of *BB* of *Flock* C which may have been ill, showed little or no decrease as compared with controls.

As in our previous studies, size of comb was measured in millimeters and is expressed as the length of the blade plus the height of the most vertical spike over the eye which was serially numbered to insure measurement of the same spike throughout. The reactions of the comb to thyroxin were complicated by the variability of other responses. With the highest doses used (*series 1*), the comb consistently showed a rapid and decided decrease in size and became dried. Complete inhibition of egg laying indicated that the gonads were also affected. Domm (31) has reported reduction in testes and even complete sterility in hyperthyroid cocks. Lower doses at greater intervals produced a similarly rapid decrease in size of comb in a few hens, which either received fairly high doses as did *PP* of *Flock E*, or proved especially sensitive to thyroxin as did *RG* of *Flock B 1, 2*.

The comb of all thyroxin treated birds in *series* 3 and 3S increased slightly in size or did not change. The average increase in length plus height in 14 of these was 14.4 with a standard error of ± 2.7 by or near the end of injections. Meantime 10 controls showed a similar increase of 6.7 ± 2.6. The difference is not significant. This dosage did not affect rate of egg production and as previously recorded, these hens also showed no increase in social success that we could detect by the methods used.

DISCUSSION

The results obtained from the present study of the social interactions of hens while some of them were being injected with varying amounts of thyroxin again emphasize the stability of their social structure. With the exception of the extreme instances from *series 1*, where dosage was large enough to produce a severe moult in short order, there was no loss of social position either among treated or untreated

hens; and this despite the fact that some of the birds which received thyroxin lost as much as 31% of their body wieght and that there was, in several instances, a depression or even a complete inhibition of egg prduction. It is true, however, that aggressiveness or fighting or bluffing ability, one or all, were decreased, as measured by success in initial encounters with strange hens, for those birds which received doses of thyroxin which were intermediate in their general physiological effects.

It may be that certain effects of hormone treatment on social status could be measured more delicately if made on birds like pigeons or doves in which the social order is based on peck dominance (4, 12) rather than on peck right as in hens. In the latter, a shift once obtained is more convincing and fewer cases are needed to be significant. With the former, a change in the asymmetry of the dominance-subordination pattern is more easily initiated but more difficult to interpret, since such a shift will more readily occur as a result of influences other than those which are under controlled experimentation.

Moulting is a normal process in the life of a bird and is customarily accompanied by changes which approach in severity many of the artificially produced moults which we have been considering. Since no reversals of social position occurred in our flocks during the entire period from October 1, 1938 to October 1, 1939 which involved only untreated, normal, healthy hens, it is evident that the social organization in homogeneous flocks of mature individuals is usually stable enough to withstand the viscissitudes of the normal moult. Even so, in experimentally staged first encounters, the condition as regards moulting is one of the factors to be considered in estimating the probable winner of a given encounter.

We have one case which indicates that normal moulting may at times be a factor in the social order of hens. On December 6, 1937, GY, an 8 months old pullet of *Flock A* 4, lost her peck right over *RG* which stood two ranks below her socially. At the time GY was moulting severely and had recently dropped her entire tail. She was not laying and was the lightest bird in her flock. *RG* was a heavy hen in full egg production. The tendency of members of a flock to moult at the same time minimizes the possibility of a disruption of an established social order as a result of changes which accompany the moult.

All our information to date indicates that the positive results given by treatment with testosterone propionate (15) are not attributable merely to general metabolic effects of the male hormone, but that the latter acted through some other channel in causing low ranking hens to advance in social status. Conversely, thyroxin, which is known to increase metabolism does not thereby make the hens more successful. If it does stimulate the normal gonads to produce more androgen, the amount is insufficient to be effective in changing the social behavior which we are studying.

We have one instance of a hen which received both testosterone propionate and thyroxin at the same time. This was YY, the alpha hen of *Flock B* 1, 2. As shown in table 1, YY was given an initial dose of 2 mg. of thyroxin followed, at 4-day intervals, by 3 injections of 3 mg. each. At the same time she was receiving daily injections of 1.25 mg. of testosterone. *BW*, which ranked second in the flock, was given similar thyroxin treatment and so serves, in part, as a control. Within 3 days after the last injection of thyroxin, YY won all of her 8 staged encounters with heavier and more powerful hens from other flocks of which she had previously beaten 6 while under the influence of testosterone alone. *BW* lost all of her fights with the same opponents although she had beaten two of them before her thyroxin treatment. Evidently the addition of thyroxin did not destroy and perhaps did not even lessen the effectiveness of YY's testosterone, and it certainly did not increase the success of *BW*.

The moult in the case of ♀♀ was somewhat less severe than that induced in *BW* and may also have been slightly delayed by the joint treatment with the androgen. Others (30) have found that androsterone inhibited the production of a hyperthyroid moult in fowls.

Our data with the lower doses of thyroxin are not adequate to prove absence or presence of an effect of thyroxin on pair-contact reactions in otherwise normal birds. We believe, however, that they do indicate that such an influence, if it exists, cannot be very marked. In the case of birds suffering from actual thyroid deficiency, the effect would probably be different, since the thyroids seem to be necessary to support the gonads in the fowl (32).

In using thyroxin with fowls, one is working with a double-edged instrument. On the one hand following such treatment, there is the demonstrated effect of an increased rate of metabolism and certain other changes, including, with certain dosages, perhaps increased gonadal activity, which might well increase chances of social success. On the other hand, there is the danger of initiating a debilitating moult. With some doses, the experimenter may be witnessing the result of a combination of these two tendencies.

For the purpose of the present analysis of the physiological factors which underly the social organization of flocks of hens, it was necessary to make an actual test of the effectiveness of increased amounts of thyroxin upon winning and holding high social rank. The depressing or indifferent results which depend on the dosage, are not surprising.

SUMMARY

Thyroxin, injected into individuals from different social levels in flocks of White Leghorn hens, produced no change in social status so long as the treatment did not initiate the combination of changes that accompany severe moulting.

Thyroxin had no noticeable influence on success in initial pair contacts with strange hens when the amount given was relatively small and produced no other readily observable, or only general effects.

Thyroxin treatment markedly decreased such successes when the hens received doses which soon produced a decided moult with accompanying physiological changes.

The largest doses used induced a sudden and complete moult and resulted in the loss of social position of 2 high ranking hens.

In addition these larger doses caused a loss of body weight, inhibition of laying, decrease in size of comb, and a general lessening of activity.

In this series of reports, we have presented evidence that epinephrine has no effect upon social status in flocks of hens; that testosterone propionate strongly increases successful social aggressiveness; that estradiol does not produce such striking results but tends to act, perhaps indirectly, in the opposite direction, and the same result has just been reported for the larger doses of thyroxin. The way is now opened for similar studies on hormones produced by the hypophysis.

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STUDIES ON THE PREGNANCY OF VIVIPAROUS SNAKES

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IN A PREVIOUS REPORT we (L. F. and T. M.) (1), published observations which were made on the ovaries of Brazilian snakes: oviparous, *Xenodon merremii;* ovoviviparous; *Crotalus terrificus*, *Bothrops jararaca* and *Bothrops alternata*. In viviparous snakes we found typical corpora lutea equal in number to the fixed embryos in the uterus. These corpora lutea do not exist in oviparous snakes and are absent whenever viviparous snakes are not pregnant. We report here results of studies on functional significance of the organ and on the migration of the egg.

From August 1936 to December 1937 we operated on 165 snakes in the Butantan Institute of São Paulo. During August very few snakes are pregnant and only after October does one find numerous cases of intra-uterine pregnancy. Eggs, the size of walnuts, are found in the ovary at the side of the more or less ripe follicles. None of the formations show an area germinativa which is only found in the eggs which have passed through the tube. The area germinativa is found on the mesometral side of the uterus and is recognizable by its transparency.

As soon as the eggs have reached the uterus, there appear in the ovaries, at the side of the existing follicles, corpora lutea in equal numbers as the embryos fixed in the uterus (see illustration in previous published work). The two ovaries are unequal in length and are situated at different distances in relation to the head.

Each of the two ovaries is completely enclosed in a sack of fine connective tissue, the stigmas of the corpora lutea remaining situated on the external side of this sack.

The tube begins at the uterus as a ribbon 1 to 2 mm. in diameter, which immediately widens in the form of a wavy ribbon 6 to 7 mm. in diameter. Opening the infundibulum with pincers or filling the tube by injection of a physiological saline solution, the opening may be expanded to the size of a hazel nut. During the period of migration of the eggs from the ovary to the carrying sack we found in numerous cases an ascites surrounding the uterus and the tube. This occurs by means of peritoneal fold (duplicature) which completely enfolds the internal sexual organs. The impression is not gained that this is a pathological accumulation of liquid, but it is probable that it is a physiological accumulation which exists only during several hours while the eggs are passing from the ovary to the uterus. This liquid mass may be the explanation of the ease with which eggs, the size of walnuts, are able to pass so easily and rapidly through a carrying tube with such a narrow anterior part.

In all cases the migration of the eggs takes place in a closed cavity and loose eggs are never found in the abdominal cavity.

We saw eggs in progress during operations on a badly anesthetized animal (*Cascavel 135*, record given below) and believe therefore that the highly muscular abdominal wall may contribute to the progress of the eggs by pressure forward.

Cascavel 135 (anesthesia with nembutal completed by ether). The laparotomy showed the bursa ovarica to be full of liquid and very much distended, the ovaries holding many corpora lutea and the tube containing eggs in its interior. The autopsy made 3 days later showed that all the eggs were placed in the uterus, in spite of the fact that the bursa was open in two places which were repaired.

Experiments made with poisonous snakes were extremely difficult since, after their poison was removed and they were confined in cages, they refused all nourish-

ment and, at the latest, died 10 days after the operation. Artificial feeding did not change this state of affairs. The autopsy showed that the field of the operation was completely without reaction. The anesthetic was also not at fault since unoperated animals as well as animals operated on with the anesthetic die after about the same period as those operated without anesthetic.

Technically, interventions in the ovaries are difficult because they are propelled outwards by active pressure movements. In spite of these difficulties, we learned after a certain time to find the ovaries by means of a small abdominal incision. In order to find the ovaries, the immediately visible, white, filiform, suprarenal glands which run closely parallel with the ovaries at a length of about 6 cm., may be taken as a point of reference. Microscopically, they consist of a continuous, typically endocrine tissue, while the ovary itself does not possess other endocrine tissue besides the corpora lutea, so that all trace of the so-called interstitial gland is lacking.

After separating the bursa ovarialis, we were able to extract the ovaries without hemorrhage and without touching the egg chambers. In this way we learned to 'deluteinize' the ovaries in the manner described by us in mammals. We were able to press out separately and entirely the corpora lutea without damaging the rest of the ovary and without breaking open the adjacent follicles.

We extracted the corpora lutea by separating them from their envelopment in one ovary or in both (uni- or bilateral 'deluteinization').

After all the corpora lutea are taken out, the eggs do not develop further and the snakes are never born alive. However, in one case, *Cascavel 26* which gave birth on December 29, 1936, one day after the operation, half of the snakes were born alive and some of them were still alive 3 days after the death of the mother. In all the rest of the animals we did not observe any progress or development of pregnancy and only saw rare cases of abortions and in some cases, regression with reabsorption of the egg. During the course of the operations we saw some cases of spontaneous regression of eggs at the side of the well-developed eggs.

Pathological corpora lutea are also found, which perhaps correspond to cystic corpora lutea in mammals and also pathologically increased and persistent follicles. In the record below, we set forth a case of unilateral 'deluteinization.'

Jararaca 156 (anesthesia by ether, very good). One ovary was easily 'deluteinized' by means of a very small abdominal incision. Only one egg chamber fell forward while the others were not even touched. The operation was performed on December 22, 1937 and the snake died on December 30, 1937. The autopsy showed that the eggs on the side, on which the ovary was 'deluteinized,' which were previously the size of a plum with a well-defined area germinativa, were reduced in number and highly pathological, while the others, on the side on which the ovary contained corpora lutea, were unaltered in size, number and aspect and contained clearly visible areas germinativas.

Poisonous snakes, under the circumstances in which they are kept in Butantan, are not especially appropriate for these experiments. However, we believe that we can here make the following resume: as we showed in the first part of our communication, corpora lutea are formed in viviparous pregnant snakes only; and as we showed in the second, more difficult part of our work, these corpora lutea have the same function as the corpora lutea in mammals, that is, to protect pregnancy until a well advanced period.

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CLINICAL AND LABORATORY NOTES

LIVER DAMAGE AND ESTROGEN INACTIVATION*

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SEVERAL INVESTIGATIONS have demonstrated that the mouse and rat liver inactivate estrogen in vitro.[1-4] Talbot[4] showed that in immature rats the administration of sublethal, liver damaging doses of carbon tetrachloride led to a degree of uterine growth that could be attributed to the failure of the damaged livers to inactivate endogenous estrogen. We have endeavored to ascertain whether or not the administration of liver damaging doses of CCl_4 to spayed females would disclose the presence of extra-gonadal estrogen or make more effective administered estrogen.

Talbot[4] administered equal parts of CCl_4 and ethyl alcohol by gavage in immature rats. We have tested two other methods in mature animals but find that only by gavage can lethal effects be had with relatively small doses. Data on the administration of various dosages of pure CCl_4 by mouth, subcutaneously injected and by gavage are presented in table 1. They demonstrate that by stomach tube doses of 2.0 cc. per kg. are partially lethal and doses of 6.0 cc. per kg. and over are completely lethal whereas doses up to 4.6 cc. per kilogram by mouth and 5.0 cc. per kilogram subcutaneously lead to no fatalities.

TABLE 1. THE TOXICITY OF CARBON TETRACHLORIDE BY VARIOUS ROUTES OF ADMINISTRATION IN OVARIECTOMIZED RATS

Dosage of CCl_4 cc. per kilogram	Route of administration					
	Subcutaneous		By mouth		By gavage	
	Number of rats	Number dead	Number of rats	Number dead	Number of rats	Number dead
1.3	1	0				
1.7	1	0				
2.0					29	15
					32*	6*
2.2	1	0				
3.5	5	0				
3.7			5	0		
4.0					3	2
4.6			8	0		
5.0	8	0				
6.0					3	3
8.0					5	5
10.0					2	2
12.0					2	2

* Animals of strain B, all others strain A.

Rats of two strains were used and the following procedures employed: The animals were ovariectomized and primed with 1.5γ of estrone in olive oil in 3 subcutaneous injections

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in 8 hours at 4 to 5 days after ovariectomy. The number of positive smears occurring on the day of priming were noted. All animals showing positive vaginal smears at 48 hours after the first priming injection (after the criteria of Smith and Smith[5]) were injected, on the 5th to 6th day after the priming injection, with 0.5γ of estrone and the number of positive smears noted. The negative rats were then primed again and on the 5th to 6th day after the priming injection those that had showed positive smears were given 2.0 cc. per kg. of CCl_4 by gavage under brief ether anaesthesia. They were then divided into two groups, one of which received 0.5γ of estrone in oil in 3 injections 24 hours after the CCl_4 administration, the other received no injection. Smears were taken at 48 hours after the first estrone injection.

The data for the two strains are given in Table 2.

TABLE 2. THE OCCURRENCE OF POSITIVE ESTROUS VAGINAL SMEARS IN SPAYED RATS BEFORE AND AFTER THE ADMINISTRATION OF 2.0 CC. CCl_4 PER KILOGRAM BY GAVAGE

Strain	Vaginal smears before priming			Vaginal smears after administration of 0.5γ estrone			Vaginal smears after administration of CCl_4					
							No estrone			0.5γ estrone		
	Number of rats	Number positive	% positive	Number of rats	Number positive	% positive	Number of rats	Number positive	% positive	Number of rats	Number positive	% positive
A	68	2	2.9	31	3	9.7	15	4	26.6	11	6	54.5
B	35	0	0.0	30	9	30.0	13	0	0.0	11	9	81.8

The increase in the number of positive smears in the strain of animals receiving only CCl_4 is not statistically significant. There was no occurrence of positive smears in the similarly treated strain B animals. Accordingly we may conclude that the administration of CCl_4 does not yield evidence of an endogenous extra-ovarian source of estrogen in spayed rats.

Despite the small numbers involved the increase in positive smears in both strain A and strain B animals receiving CCl_4 and 0.5γ estrone is statistically significant ($P<0.01$) when comparison is made with the control groups receiving 0.5γ estrone alone. We may conclude that liver damage by CCl_4 results in a decreased inactivation of administered estrone. Our data on the dosage-response curve of strain A indicate that the 54.5% positive observed lies at the 0.9γ level giving an 80% increase of activity of the administered estrone by the CCl_4 administration. We do not have a similar dosage-response curve for strain B but the indications are that it is more responsive to estrone than strain A so that the 81.8% positive in the CCl_4 animals probably represents a roughly similar increase.

Emmens[6] has reported the presence in urines of inactive materials that enhance estrogen activity up to ten times. Conceivably these materials might be liver-damaging substances but our evidence for only an 80% increase in the activity of estrone after the administration of CCl_4 would militate against such a possibility.

SUMMARY

Data on carbon tetrachloride administered to 16 ovariectomized rats subcutaneously, 13 by mouth and to 76 by gavage indicate that the latter is the most effective lethal and sublethal route. When a sublethal dose of carbon tetrachloride was administered to 15 ovariectomized rats of one strain and 13 of another no significant increase in the percentage of positive smears occurred. The administration of 0.5γ of estrone subcutaneously after carbon tetrachloride by gavage led to a significant increase over controls of the percentage of positive smears in 11 rats of one strain and in 11 rats of a second strain. The effectiveness of the administered estrone was increased by about 80% in strain A. It is concluded that liver damage by carbon tetrachloride does not reveal the presence of endogenous extraovarian estrogen in spayed animals but does increase the effectiveness of administered estrogen presumably by interfering with detoxification.

We are indebted to Dr. Erwin Schwenk of the Schering Corporation for the crystalline estrone employed in these studies.

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INDUCTION OF SEXUAL RECEPTIVITY IN ESTROGEN CONDITIONED SPAYED FEMALE GUINEA PIGS BY ORALLY ADMINISTERED PROGESTERONE AND PREGNENINOLONE*

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PREVIOUS INVESTIGATIONS of the effectiveness of orally administered progesterone and progesterone-like compounds have demonstrated the necessity of larger doses if results comparable to those produced by subcutaneous injection are to be obtained. In the rabbit the minimal oral dose of corpus luteum hormone necessary to produce distinct progestational proliferation is 50 rabbit units.[1] Even this amount did not produce as marked an effect as 1 RB. U. injected subcutaneously. Hohlweg and Inhoffen[2] were unable to detect any alterations in the uteri of rabbits when as much as 60 mg. progesterone was given orally over a 5-day period. This amount represents 100 times the effective subcutaneous dose. On the other hand, they were successful in producing progestational changes with orally administered pregneninolone in quantities (4 mg.) twice that necessary by injection. Clauberg and Ustün[3] also report that pregneninolone (proluton-C) is effective when given orally to humans in 6 times the intramuscular dosage.

TABLE 1. INDUCTION OF RECEPTIVITY BY ORALLY ADMINISTERED PROGESTERONE AND PREGNENINOLONE

No. of animals	Quantity	No. in heat	% in heat	Latent period		Length of heat		Combined latent and length
				Mean	Range	Mean	Range	
	mg.							
Progesterone								
3	15.0	3	100.0	3.0	2.5– 3.5	8.6	6.5–10.0	11.6
2	10.0	2	100.0	3.8	3.5– 4.0	6.5	5.5– 7.5	10.3
3	5.0	3	100.0	3.5	3.5– 3.5	7.7	7.5– 8.0	11.2
3	4.0	3	100.0	3.7	3.5– 4.0	8.5	6.5– 9.5	12.0
4	2.0	4	100.0	4.4	4.0– 5.0	8.4	7.5– 9.5	12.8
14	1.5	14	100.0	4.6	3.5– 5.5	7.0	4.5– 8.5	11.6
2	1.4	2	100.0	4.8	4.0– 5.5	4.3	2.0– 6.5	9.1
7	1.3	7	100.0	4.4	4.0– 5.5	4.4	4.0– 5.5	8.8
10	1.2	9	90.0	5.3	3.5– 7.5	6.1	4.5– 8.5	11.4
7	1.1	6	85.7	4.8	3.0– 8.0	7.2	4.5–10.0	12.0
13	1.0	11	84.6	3.8	2.0– 6.0	7.7	6.0–11.0	11.5
11	0.8	6	54.5	4.3	2.5– 5.5	3.6	1.5– 6.5	7.9
9	0.6	5	55.5	4.4	3.0– 6.0	4.4	3.5– 5.5	8.8
8	0.4	3	37.5	4.3	3.5– 6.0	3.0	2.0– 3.5	7.3
18	0.2	3	16.7	4.8	4.0– 5.0	5.0	3.0– 7.5	9.8
Pregneninolone								
8	2.0	8	100.0	4.2	3.8– 5.0	9.4	6.8–10.8	13.6
16	1.0	10	62.5	5.0	2.5–11.0	6.1	2.5–10.0	11.1
Control injections of progesterone								
7	0.05	6	85.9	7.8	5.0–12.0	5.8	1.5– 8.0	13.6
9	0.1	8	88.8	4.9	4.0– 6.3	6.9	3.0–10.0	11.8

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An explanation for the refractiveness of the animal organism to orally administered corpus luteum extract has been advanced by Engelhart[4] who showed that the effective hormone is split in vitro and rendered ineffective by the pancreatic enzyme, lipase. This applies particularly to the corpus luteum extract whereas the purified crystalline hormone, progesterone, is more resistant.[2]

Using the induction of sexual receptivity in spayed female guinea pigs as a criterion of effectiveness,[5] the action of progesterone (proluton, Schering)‡ given in capsules and pregneninolone tablets administered orally has been studied. The animals were observed at half-hourly or hourly intervals to determine the length of the latent period (interval between feeding and beginning of heat reaction) and heat.

The effects of graded doses of progesterone and pregneninolone are summarized in table 1. It is apparent that 1 mg. of progesterone was sufficient to induce normal copulatory behavior in a high percentage of animals previously conditioned with subcutaneously injected estrogen. Although lower doses were also effective, the percentage of animals responding was smaller. The response which followed administration of the smallest quantity, 0.2 mg., could have been caused by the injected estrogen. Pregneninolone was effective in 8 of 8 animals when 2 mg. were given and in 10 of 16 animals when 1 mg. was given.

Inasmuch as 0.05 mg. progesterone injected subcutaneously is sufficient to induce heat in a large percentage of animals,[6] it appears that when the induction of heat in the female guinea pig is the end-point, the ratio of orally administered to injected progesterone is about 20 : 1. This ratio is considerably lower than that of 50 : 1 obtained by Portman[1] when progestational changes in the rabbit uterus were used as the end-point.

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‡ Grateful acknowledgment is made to Dr. Erwin Schwenk of the Schering Corporation for the progynon-B and proluton, and to Dr. Ernst Oppenheimer of the Ciba Pharmaceutical Products, Inc., for the pregneninolone tablets.

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ACETYLCHOLINE-EQUIVALENT CONTENT OF THE VAGINA AND VULVA OF OVARIECTOMIZED RABBITS, BEFORE AND AFTER ADMINISTRATION OF ESTROGEN*

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THE FOLLOWING DATA were obtained in connection with the observation that estrogen increases appreciably the concentration of free acetylcholine in the uterus of rabbits[1,2] and in the nasal mucosa of ovariectomized rabbits and cats.[3] The method of extraction and testing the tissues for this substance was the same as in earlier experiments; it was, namely, that of Chang and Gaddum,[4] modified slightly as described elsewhere.[1] The rabbits were ovariectomized well in advance of the experiment, usually 18 to 21 days previously. At the time of the experiment, two rabbits were anesthetized with dial (Ciba) given intramuscularly. The tissues of one were taken without further treatment, those of the other, at the end of one hour after subcutaneous injection of estrogen (500γ of estradiol-dipropionate,

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di-ovocylin, Ciba; 0.2–2.8 mg. estradiol-benzoate, progynon-B, Schering; and 10,000 R.U. amniotin, Squibb). Twelve untreated, ovariectomized rabbits were used. Vulval tissue was taken from 13 rabbits treated with estrogen, and extracts of vaginal tissue were prepared from 16 estrogen injected rabbits.

The data summarized in figure 1 show the values obtained in single experiments (dots) and the mean values for each group are indicated by the shaded bars. For comparison, the average concentrations of acetylcholine found in the rabbit uterus in previous experiments[2] are also shown. It will be observed that no cholinergic effect of estrogen was demonstrable

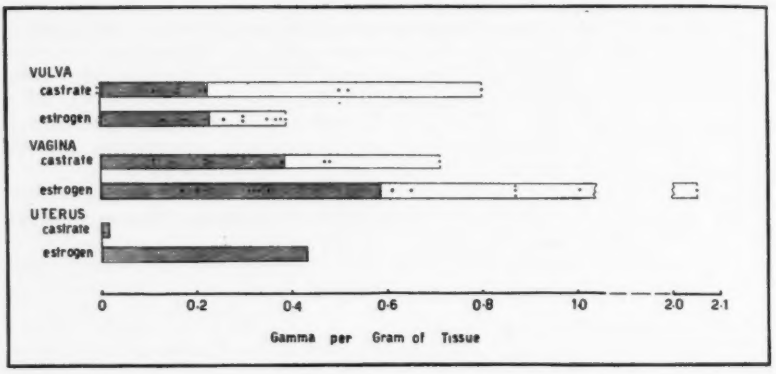

Fig. 1.

in the vulva, as it clearly is in the uterus, and as it appears to be in the vagina. The concentration of an acetylcholine-like substance in the vagina and vulva from untreated rabbits exceeds that found in uteri of the same animals. The large amount of nervous tissue, especially in the vagina, may be a contributing factor to this, although its real basis is not known.

Mention may be made of the fact that measurements were made of the acetylcholine-equivalent content of intestine, skeletal muscle and spleen in these rabbits. The values for these tissues agreed roughly with those cited by Chang and Gaddum,[3] and were unaffected by injection of estrogen.

Summary. Comparison is made of the concentration of an acetylcholine-like substance in the vulva and vagina of untreated, ovariectomized rabbits, and of estrogen injected ovariectomized rabbits. No effect of estrogen on the concentration of such a substance could be shown in the vulva, although some effect in this regard was demonstrable in the vagina. The cholinergic effect was less in the latter tissue, however, than it is in the uterus, as shown by earlier experiments.